

IN THE UNITED STATES DISTRICT COURT  
FOR THE DISTRICT OF DELAWARE

NIPPON SHINYAKU CO., LTD.,	)	
	)	
Plaintiff,	)	
	)	
v.	)	C.A. No. 21-1015 (GBW)
	)	
SAREPTA THERAPEUTICS, INC.,	)	<b>VOLUME 3 (Exhibits 34-53)</b>
	)	
Defendant.	)	

---

SAREPTA THERAPEUTICS, INC.,	)	
	)	
Defendant and Counter-Plaintiff,	)	
	)	
v.	)	
	)	
NIPPON SHINYAKU CO., LTD.	)	
and NS PHARMA, INC.	)	
	)	
Plaintiff and Counter-	)	
Defendants.	)	

**JOINT APPENDIX TO CLAIM CONSTRUCTION BRIEF  
FOR THE WILTON/UWA PATENTS**

MORGAN, LEWIS & BOCKIUS LLP  
Amy M. Dudash (#5741)  
1201 North Market Street, Suite 2201  
Wilmington, DE 19801  
(302) 574-3000  
amy.dudash@morganlewis.com

*Attorneys for Plaintiff/Counterclaim  
Defendant Nippon Shinyaku Co., Ltd. and  
Counterclaim Defendant NS Pharma, Inc.*

MORRIS, NICHOLS, ARSHT & TUNNELL LLP  
Jack B. Blumenfeld (#1014)  
Megan E. Dellinger (#5739)  
1201 North Market Street  
P.O. Box 1347  
Wilmington, DE 19899  
(302) 658-9200  
jblumenfeld@morrisnichols.com  
mdellinger@morrisnichols.com

*Attorneys for Defendant/Counter-Plaintiff  
Sarepta Therapeutics, Inc.*

OF COUNSEL:

Amanda S. Williamson  
Christopher J. Betti  
Krista V. Venegas  
Maria E. Doukas  
Zachary Miller  
Guylaine Haché  
Michael T. Sikora  
MORGAN, LEWIS & BOCKIUS LLP  
110 North Wacker Drive, Suite 2800  
Chicago, IL 60601  
(312) 324-1000

Eric Kraeutler  
MORGAN, LEWIS & BOCKIUS LLP  
1701 Market Street  
Philadelphia, PA 19103  
(215) 693-5000

OF COUNSEL:

Charles E. Lipsey  
J. Derek McCorquindale  
Ryan O'Quinn  
FINNEGAN, HENDERSON, FARABOW,  
GARRETT & DUNNER, LLP  
1875 Explorer Street, Suite 800  
Reston, VA 20190-6023  
(571) 203-2700

William B. Raich  
Michael J. Flibbert  
Yoonhee Kim  
Yoonjin Lee  
FINNEGAN, HENDERSON, FARABOW,  
GARRETT & DUNNER, LLP  
901 New York Avenue, NW  
Washington, DC 20001-4413  
(202) 408-4000

Alissa K. Lipton  
FINNEGAN, HENDERSON, FARABOW,  
GARRETT & DUNNER, LLP  
Two Seaport Lane  
Boston, MA 02210-2001  
(617) 646-1600

March 20, 2023

# EXHIBIT 34

Lancet neurology.  
v. 8, no. 10 (Oct. 2009)  
General Collection  
W1 LA453U  
2009-10-22 13:03:33

# ET Neurology

Volume 8 · Issue 10 · October 2009

www.thelancet.com/neurology



PROPERTY OF THE  
NATIONAL  
LIBRARY OF  
MEDICINE

## Articles

Restoration of dystrophin in Duchenne muscular dystrophy: a proof-of-concept study

See page 929

## Review

Dopamine agonists in Parkinson's disease: risks and benefits

See page 929

Impact Factor  
14.27

**The Lancet Neurology**

32 Jamestown Road  
London NW1 7BY, UK  
T +44 (0) 20 7424 4221  
F +44 (0) 1865 853014  
helen.frankish@lancet.com

www.thelancet.com/neurology

**Editor**

Helen Frankish

**Deputy Editor**

Elena Becker-Barroso

**Senior Editors**

Heather Brown  
Alison Rowan

**Senior Assistant Editor**

Steven Goodrick

**Assistant Editor**

Jessica Knights

**Web Editors**

Richard Lane  
Erika Niesner

**Web Content Coordinator**

Andrew Kyriacou

**Editorial Board**

Marie-Germaine Bousser (France)  
Alexis Brice (France)  
Paolo Calabresi (Italy)  
Hans-Christoph Diener (Germany)  
Geoffrey Donnan (Australia)  
Giovanni Frisoni (Italy)  
Francesc Graus (Spain)  
Jes Olesen (Denmark)  
Emilio Perucca (Italy)  
Chris Polman (Netherlands)  
Niall Quinn (UK)  
Olivier Rascol (France)  
Peter Rothwell (UK)  
Richard Rudick (USA)  
Ley Sander (UK)  
Lon Schneider (USA)  
Tom Solomon (UK)  
Jon Stoessl (Canada)  
William Theodore (USA)  
Alan Thompson (UK)  
Steven Warach (USA)  
Peter Whitehouse (USA)  
Max Wizenitz (USA)  
John Wokke (Netherlands)

**Editorial Consultant**

Peter Rudge (UK)

**Publisher/Editorial Director**

Richard Horton

**Information for Authors**

Guidelines for authors are published  
online at [www.thelancet.com/authors/  
neurology/authorinfo](http://www.thelancet.com/authors/neurology/authorinfo)

THE LANCET® is a registered  
trademark of Elsevier Properties  
SA, used under licence.

**Edition**

UK

**Ombudsman**

Charles Warlow (c/o The Lancet  
or [ombudsman@lancet.com](mailto:ombudsman@lancet.com))

**Cover**

Local restoration of dystrophin  
expression in Duchenne muscular  
dystrophy (see page 918)



Tim Marrs/Central Illustration Agency  
(<http://www.centralillustration.com>)

**Leading Edge**

- 869 Pandemic influenza: a priority for the neurological community

**Reflection and Reaction**

- 870 Trials of diminishing relapses for diminishing returns  
A Coles
- 871 Poor outcomes after endovascular treatment of symptomatic carotid stenosis: time for a moratorium  
P M Rothwell
- 873 Less is more: therapeutic exon skipping for Duchenne muscular dystrophy  
A Aartsma-Rus, G-J B van Ommen
- 875 Cervicogenic headache: a pain in the neck for some neurologists?  
P J Goadsby
- 877 Can the WATCHMAN device truly PROTECT from stroke in atrial fibrillation?  
B J Wrigley, G Y H Lip

**Correspondence**

- 879 Epidural abscesses in Africa  
T B Rabin, G Pradilla, D Rigamonti
- 880 Quality of life in children with CMT type 1A  
S Ramchandren and others; J Burns

**In Context**

- 882 When a patient chooses death: divided attitudes  
K Stone
- 884 Profile  
Paul O'Connor: from manual labour to multiple sclerosis mastery  
R Williams
- 885 Ten most wanted
- 885 Lifeline  
M M Brown
- 886 Book  
Unravelling the mysteries of juvenile Huntington's disease  
F Cendes
- 886 Book  
Navigating neuroradiology  
C Croteau, E C Bourekas
- 887 Book  
Unveiling the less explored side of Parkinson's disease  
M Merello
- 888 News in brief

**Articles**

- 889 250 µg or 500 µg interferon beta-1b versus 20 mg glatiramer acetate in relapsing-remitting multiple sclerosis: a prospective, randomised, multicentre study  
P O'Connor and others
- 898 Endovascular treatment with angioplasty or stenting versus endarterectomy in patients with carotid artery stenosis in the Carotid And Vertebral Artery Transluminal Angioplasty Study (CAVATAS): long-term follow-up of a randomised trial  
J Ederle and others
- 908 Long-term risk of carotid restenosis in patients randomly assigned to endovascular treatment or endarterectomy in the Carotid and Vertebral Artery Transluminal Angioplasty Study (CAVATAS): long-term follow-up of a randomised trial  
L H Bonati and others
- 918 Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study  
M Kinali and others

**Review**

- 929 A reassessment of risks and benefits of dopamine agonists in Parkinson's disease  
A Antonini and others
- 938 Blood pressure as a prognostic factor after acute stroke  
V Tikhonoff and others
- 949 Malignant middle cerebral artery infarction: clinical characteristics, treatment strategies, and future perspectives  
H B Huttner, S Schwab
- 959 Cervicogenic headache: an assessment of the evidence on clinical diagnosis, invasive tests, and treatment  
N Bogduk, J Govind

➔ Peer reviewed and fast-tracked to publication in 4–8 weeks

Ⓜ Previously published online

✱ See [www.thelancet.com](http://www.thelancet.com) for WebExtra content

This material may be protected by Copyright law (Title 17 U.S. Code)

- 9 Lewis SC, Warlow CP, Bodenham AR, et al, for the GALA Trial Collaborative Group. General anaesthesia versus local anaesthesia for carotid surgery (GALA): a multicentre, randomised controlled trial. *Lancet* 2009; **372**: 2132-42.
- 10 Bond R, Rerkasem K, Rothwell PM. A systematic review of the risks of carotid endarterectomy in relation to the clinical indication and the timing of surgery. *Stroke* 2003; **34**: 2290-301.
- 11 Rothwell PM, Eliasziw M, Gutnikov SA, Warlow CP, Barnett HJM, for the Carotid Endarterectomy Trialists Collaboration. Effect of endarterectomy for symptomatic carotid stenosis in relation to clinical subgroups and to the timing of surgery. *Lancet* 2004; **363**: 915-24.
- 12 Rothwell PM, Z Mehta, SC Howard, SA Gutnikov, CP Warlow. From subgroups to individuals: general principles and the example of carotid endarterectomy. *Lancet* 2005; **365**: 256-65.
- 13 Hobson RW 2nd. Update on the Carotid Revascularization Endarterectomy versus Stent Trial (CREST) protocol. *J Am Coll Surg* 2002; **194**: 59-14.

## Less is more: therapeutic exon skipping for Duchenne muscular dystrophy



Duchenne muscular dystrophy (DMD) is a lethal X-linked progressive muscle-wasting disease caused by premature truncation of the translation of DMD mRNA into dystrophin. Owing to improved respiratory support and treatment with steroids, patients can now survive into early adulthood, which is one or two decades longer than they could survive without such interventions. Despite much research worldwide, no disease-modifying treatment is yet available; however, promising results are being achieved with antisense oligonucleotides. Antisense oligonucleotides are modified DNA or RNA analogues that hybridise with a target DNA or RNA sequence, and they can be designed to prevent specific exons of DMD from being spliced into the mRNA. This so-called exon skipping can reframe the disrupted open reading frame of DMD to produce a shorter but functional protein (figure), such as the protein produced in individuals with the milder form of the disease, Becker muscular dystrophy.<sup>1</sup> This less is more approach has been developed over the past decade in patient-derived cell cultures and in the *mdx* mouse model.<sup>2-5</sup>

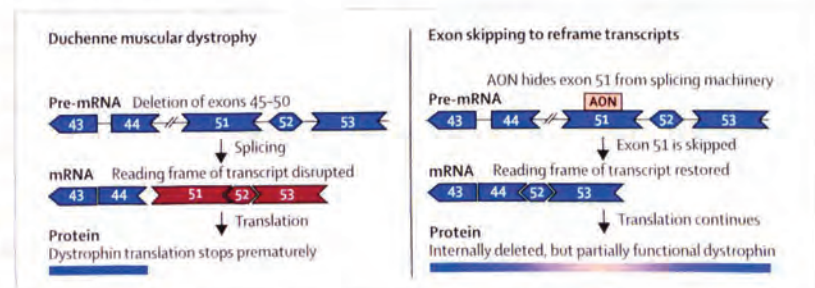
Modifications to the chemistry of antisense oligonucleotides are necessary to increase their half life and prevent the cleavage of RNA-RNA hybrids by RNase H, which is the goal of antisense-mediated knockdown of gene function but is unwanted for antisense-mediated modulation of splicing. The most commonly used chemistries for exon-skipping antisense oligonucleotides are 2'-O-methyl RNA with a negatively charged phosphorothioate backbone (2'-OMePS) and uncharged phosphorodiamidate morpholino oligomers (PMO), because both are stable and non-toxic.

In 2007, an intramuscular proof-of-concept clinical study was reported by van Deutekom and co-

authors.<sup>6</sup> They used PRO051, a 2'-OMePS RNA that targets exon 51. Skipping of exon 51 corrects several mutations in the DMD deletion "hotspot", which amounts to about 13% of the mutations that cause DMD. In the September issue of *The Lancet Neurology*, Kinali and co-authors<sup>7</sup> report the results of a similar proof-of-concept study with intramuscular AVI-4658, a PMO that also targets exon 51. Although there are similarities and differences between the two studies, the main combined messages are that there are no drug-related adverse effects and that both backbone chemistries have about equal effectiveness. These findings are valuable because they imply that there is more freedom in the choice of chemistry than has been known so far. However, although nucleic acid-based drugs, such as PMO or 2'-OMePS, are promising because of their apparent low toxicity, and they have not yet been tested over the prolonged period of administration required for treating childhood disease.

The table compares two studies. van Deutekom and co-authors injected a 2x1 cm area of the tibialis anterior

Published Online  
August 26, 2009  
DOI:10.1016/S1474-4422(09)70229-7  
See [Articles](#) page 918



**Figure 1: Antisense-mediated exon skipping to reframe DMD transcripts**

Patients with Duchenne muscular dystrophy have mutations in DMD, the gene that encodes dystrophin. The mutations disrupt the open reading frame of dystrophin (in this example, exons 45-50 are deleted). Consequently, protein translation stops prematurely, resulting in a non-functional protein. By use of antisense oligonucleotides that target a specific exon in which there is a mutation that truncates the expression of dystrophin (exon 51 in this example), the reading frame can be restored. This enables the production of an internally deleted but partially functional dystrophin. AON=antisense oligonucleotide.

	van Deutekom and co-authors	Kinali and co-authors
Compound	PRO051	AVI-4658
Target	Exon 51	Exon 51
Length (nucleotides)	20	30
Chemistry	2'-O-methyl-ribooligo-nucleoside-phosphorothioate	Phosphorodiamidate morpholino oligomer
Sequence*	UCAAAGGAAGAUGGCAUUUCU	CTCCAACATCAAGGAAGATGGCATTCTAG
Placebo	No	Yes
Dose	0.8 mg	0.9 mg
Number of patients	4	5†
Target muscle	Tibialis anterior	Extensor digitorum brevis
Proportion of positive fibres	64–97%‡	44–79%§
Proportion of dystrophin expression	17–35%¶	22–32%  , **

\*The two antisense oligonucleotides target the same region in exon 51. AVI-4658 contains the sequence of PRO051 (underlined). The PMO is a DNA analogue, whereas 2'-OMePS is an RNA analogue, which explains why the antisense oligonucleotides contain the nucleotides thymine and uracil. †Two other patients received a lower dose (0.09 mg), which was ineffective. ‡Not corrected for positive fibres in saline injected contralateral muscle. §Corrected for number of positive fibres in saline injected contralateral muscle (0.3–5.0% positive fibres). ¶Corrected for background staining in saline injected contralateral muscle (11–21%). ||Not corrected for background staining in saline injected contralateral muscle. \*\*Up to 42% when selecting only positive fibres; this figure is not available for the other study.

**Table 1: Comparison of studies of intramuscular antisense oligonucleotides in patients with Duchenne muscular dystrophy**

muscle in the lower leg, but took only a small biopsy of the muscle 4 weeks after treatment. Kinali and co-authors targeted a 1 cm<sup>2</sup> area of the extensor digitorum brevis, a small muscle in the foot, and the muscle was completely removed for analysis; they also biopsied the saline-injected contralateral muscle. Kinali and co-authors showed that low-dose AVI-4658 did not induce expression of dystrophin that was greater than the background expression, whereas high-dose AVI-4658 induced a significant increase in dystrophin expression and the number of dystrophin-positive fibres seen with immunohistochemical staining.

The proportion of dystrophin-positive fibres and the amount of dystrophin detected vary between the studies but, owing to the differences in several parameters (eg, subtraction of expression in contralateral control muscle, the volume of muscle that is injected [and hence, the local expression], and the selection for analysis of fascicles that are adjacent to the injection track), not much significance should be attributed to these differences in results. Kinali and co-authors summarise it best when they say that “both studies reported unequivocal expression of dystrophin at similar concentrations”; moreover, both are proof-of-concept studies. The next step, which both groups are currently undertaking, is to deliver the antisense oligonucleotide systemically; therefore, detailed

comparisons of intramuscular results are largely irrelevant. Only systemic trials will reveal the true promise of this approach, and further trials are needed to validate the functional benefit, or at least the decline in disease progression. Indeed, any resolution as to which chemistry (if either) is the best choice should wait until after systemic comparisons are made, under conditions that are otherwise identical.<sup>8</sup> Notably, although delivery is the main obstacle to the clinical application of many antisense-based treatments, the pathophysiology of DMD benefits delivery,<sup>9</sup> and uptake of antisense oligonucleotides is much better in damaged, dystrophic muscles than it is in healthy muscle.

Finally, the sequence-specific approach<sup>9</sup> has implications for future personalised therapy. Although skipping of exon 51 is applicable for 13% of patients with Duchenne muscular dystrophy,<sup>10</sup> it will not benefit the other 87%, whereas skipping of 10 exons might be beneficial for more than 70% of patients with a deletion mutation in DMD, or 40% of all patients. Other approaches are being developed that could raise the number of patients who can be treated: for example, antisense oligonucleotides to duplications and point mutations through double-exon<sup>11,12</sup> and multi-exon skipping.<sup>13,14</sup> Although these approaches look promising on paper, not all are successful in practice, and we might need to learn much more about the complex splicing of this large gene (2.5 Mbp and 79 exons) to optimise its therapeutic correction.<sup>10</sup> In all cases, this process will require close coordination with regulatory agencies,<sup>9,10</sup> because there are too few patients with the rarer mutations to set up independent clinical trials.

Annemieke Aartsma-Rus, Gert-Jan B van Ommen  
Department of Human Genetics, Leiden University Medical Centre  
PO Box 9600, 2300 RC Leiden, Netherlands  
g.jvo@lumc.nl

AA-R and G-JBvO are employees of Leiden University Medical Center and coinventors on patent applications for antisense sequences and exon-skipping technology. Leiden University Medical Center has licensed the rights to the patents on PRO051 exclusively to Prosensa. The inventors specified on the patents, who include AA-R and G-JBvO, are jointly entitled to a share of any future royalties paid to Leiden University Medical Center, should the therapy eventually be brought to the market. G-JBvO is an unpaid member of the external scientific advisory board of Prosensa.

- 1 Monaco AP, Bertelson CJ, Liechti-Gallati S, Moser H, Kunkel LM. An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. *Genomics* 1988; 2: 90–95.
- 2 Aartsma-Rus A, Janson AA, Kaman WE, et al. Therapeutic antisense-induced exon skipping in cultured muscle cells from six different DMD patients. *Hum Mol Genet* 2003; 12: 907–14.

- 3 Alter J, Lou F, Rabinowitz A, et al. Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. *Nat Med* 2006; **12**: 175–77.
- 4 Mann CJ, Honeyman K, Cheng AJ, et al. Antisense-induced exon skipping and synthesis of dystrophin in the mdx mouse. *Proc Natl Acad Sci USA* 2001; **98**: 42–47.
- 5 van Deutekom JC, Bremmer-Bout M, Janson AA, et al. Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells. *Hum Mol Genet* 2001; **10**: 1547–54.
- 6 van Deutekom JC, Janson AA, Ginjaar IB, et al. Local dystrophin restoration with antisense oligonucleotide PRO051. *N Engl J Med* 2007; **357**: 2677–86.
- 7 Kinali M, Arechavala-Gomez V, Feng L, et al. Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation proof-of-concept study. *Lancet Neurol* 2009; published online August 26. DOI: 10.1016/S1474-4422(09)70211-X.
- 8 Heemskerk HA, De Winter CL, de Kimpe SJ, et al. *In vivo* comparison of 2'-O-methylphosphorothioate and morpholino antisense oligonucleotides for Duchenne muscular dystrophy exon skipping. *J Gene Med* 2009; **11**: 257–66.
- 9 Hoffman EP. Skipping toward personalized molecular medicine. *N Engl J Med* 2007; **357**: 2719–22.
- 10 Aartsma-Rus A, Fokkema IF, Verschuuren JJ, et al. Theoretic applicability of antisense-mediated exon skipping for Duchenne muscular dystrophy mutations. *Hum Mutat* 2009; **30**: 293–99.
- 11 Aartsma-Rus A, Janson AA, Kaman WE, et al. Antisense-induced multiexon skipping for Duchenne muscular dystrophy makes more sense. *Am J Hum Genet* 2004; **74**: 83–92.
- 12 Yokota T, Lu QL, Partridge T, et al. Efficacy of systemic morpholino exon-skipping in Duchenne dystrophy dogs. *Ann Neurol* 2009; **65**: 667–76.
- 13 van Vliet I, de Winter CL, van Deutekom JC, van Ommen GJ, Aartsma-Rus A. Assessment of the feasibility of exon 45–55 multiexon skipping for Duchenne muscular dystrophy. *BMC Med Genet* 2008; **9**: 105.
- 14 Aartsma-Rus A, Kaman WE, Weij R, den Dunnen JT, van Ommen GJ, van Deutekom JC. Exploring the frontiers of therapeutic exon skipping for Duchenne muscular dystrophy by double targeting within one or multiple exons. *Mol Ther* 2006; **14**: 401–07.

## Cervicogenic headache: a pain in the neck for some neurologists?

In this issue of *The Lancet Neurology*, Bogduk and Govind review the vexed topic of cervicogenic headache.<sup>1</sup> As a neurologist interested in headache, it seems self-evident that this topic should be of interest. Much of what has passed for science in this field is rightly criticised by the authors, and there seems no benefit in trawling over the arguments, for if there was no controversy there would be no issue. As the authors say, the anatomy and physiology are clear enough,<sup>2</sup> so what is the problem? There are several issues, again largely and capably discussed by Bogduk and Govind; I would like to highlight some of these from the neurologist's perspective, as I think we need to get our house in order. The three particular topics of note are: the clinical implications of the anatomy and physiology, the problem of referral bias, and the issue of primary versus secondary headache from a neurologist's point of view. Finally, how should we proceed?

Perhaps the most basic issue revolves around the anatomy and physiology of upper cervical segment nociceptive afferents and their projections to second-order neurons. In experimental animals, the cervical and ophthalmic division of trigeminal neurons clearly synapse on common second-order neurons<sup>3,4</sup> in the trigeminocervical complex.<sup>5</sup> The basic data are supported by clinical observations, such as referred pain from cervical muscles<sup>6</sup> and the C2–3 zygapophysial joint.<sup>7</sup> Interestingly, the clinical data reviewed<sup>1</sup> suggest that the caudal limit of cranial referral in the neck is at the level

of the C3 afferents; this is certainly consistent with the laboratory anatomy.<sup>8</sup> What would the convergence of these afferents predict? The data suggest that nociceptive activation in structures innervated by either trigeminal or upper cervical afferents might result in a perception of pain that is not anatomically related to the structure with the pathology. Put simply, the anatomy predicts that the simple clinical localisation process of "pain marks the spot" is doomed to fail. Moreover, the data all suggest that cervicogenic headache—headache from activation of nociceptors in the upper cervical spine—should exist.

An important aspect of the apparent controversy in this field might relate substantially to referral bias. In the USA, more than 90% of patients who present to a physician with disabling headache lasting longer than 3 months have migraine.<sup>9</sup> This predominance of migraine is likely to feed into neurology practice, which means that, whatever the prevalence of cervicogenic headache, neurological practice will be swamped with cases of migraine. If the above analysis of the anatomy and physiology is accepted, and the data on neck symptoms in patients with premonitory symptoms suggest that about 90% of these patients have these symptoms,<sup>10</sup> then most patients seen in neurology or headache practice with neck discomfort have migraine. This assumption does not mean cervicogenic headache does not exist, it does not demean it, nor imply anything negative; this is just referral bias. In a recent retrospective study of patients who responded to occipital nerve

See [Review](#) page 959

# EXHIBIT 35

## FDA Approves Sarepta Therapeutics' Vyondys 53 for Treatment of DMD Amenable to Exon 53 Skipping

[mdaquest.org/fda-approves-sarepta-therapeutics-vyondys-53-for-treatment-of-dmd-amenable-to-exon-53-skipping/](https://mdaquest.org/fda-approves-sarepta-therapeutics-vyondys-53-for-treatment-of-dmd-amenable-to-exon-53-skipping/)

December 13, 2019



On Dec. 12, the US Food and Drug Administration (FDA) granted accelerated approval to golodirsen (Vyondys 53) for the treatment of Duchenne muscular dystrophy (DMD) in patients amenable to skipping exon 53. It is the second exon-skipping, disease-modifying drug to treat DMD, the most common childhood form of muscular dystrophy. The drug is administered by intravenous infusion. Vyondys 53 will be made available in the United States and marketed by Sarepta Therapeutics.

In September 2016, the approval of eteplirsen (marketed by Sarepta as Exondys 51) marked a watershed moment for treating neuromuscular diseases with gene-targeting therapies such as exon skipping. Approval of Vyondys 53, another exon-skipping drug designed to treat a different subset of DMD individuals than those who qualify for Exondys 51, is another significant step forward in the development of therapies for DMD — and all neuromuscular diseases — that target the root cause of the disease.

“The approval of Vyondys 53 is another breakthrough toward treating Duchenne, a disease that, up until a few years ago, had no approved therapies,” says MDA’s Executive Vice President and Chief Research Officer Sharon Hesterlee, PhD. “It’s another milestone for the field of DMD research and drug development, and it represents the fulfillment of the promise of effective genetic medicines for DMD patients and their families.”

DMD is caused by mutations in the dystrophin gene (*DMD*) on the X chromosome that result in little or no production of dystrophin, a protein essential to keeping muscle cells intact. Vyondys 53 is called an “exon-skipping” drug in that it is designed to target and promote skipping over a section of genetic code in order to avoid the gene mutation and produce more of the dystrophin protein. It is estimated that up to 8% of patients with DMD have mutations amenable to treatment with Vyondys 53. Although treatment with Vyondys 53 will not cure DMD, it could slow progression of the disease, which, in turn, could extend the length of time individuals with DMD could walk, eat independently, and breathe without assistance.

### **Clinical trials support approval of Vyondys 53**

The FDA based its decision to grant accelerated approval to Vyondys 53 on the positive results of a pivotal (phase 1/2) clinical trial conducted in Europe to assess the safety, tolerability, pharmacokinetics (how the drug is absorbed, distributed, and metabolized in the body), and efficacy (dystrophin expression) of Vyondys 53 in 25 boys with DMD with confirmed deletions of the dystrophin gene amenable to exon 53 skipping. Preliminary results reported in late 2017 showed that treatment with Vyondys 53 for approximately one year was associated with a significant boost in dystrophin protein production. Muscle biopsies confirmed that all participants responded to the drug, with increased levels of exon 53 skipping compared to measurements taken at baseline (prior to beginning treatment). A 10.7-fold increase in levels of dystrophin protein was seen, equating to a rise from an initial average among patients of 0.095 percent of normal protein levels to a mean of 1.019 percent.

The 25 boys with DMD who participated in the phase 1/2 clinical trial were evaluated for a total of 144 weeks. Positive results were seen across all endpoints, including proper exon skipping, increased dystrophin production, and increased dystrophin intensity. The approval was based on an observed statistically significant increase in dystrophin production in skeletal muscle of patients treated with Vyondys 53, which is reasonably likely to predict clinical benefit for those patients. The continued approval of Vyondys 53 may be contingent on confirmation of a clinical benefit in a post-marketing confirmatory trial (ESSENCE), which is currently enrolling and expected to conclude by 2024.

Hypersensitivity reactions, including rash, fever, itchy skin, hives, dermatitis, and skin exfoliation, have occurred in patients who were treated with Vyondys 53. Renal toxicity was observed in animal studies. The most common adverse reactions that occurred in at least 20% of treated patients and more frequently than in placebo-group patients were headache (41%), fever (41%), fall (29%), abdominal pain (27%), nasopharyngitis (27%), cough (27%), vomiting (27%), and nausea (20%).

### **About SareptAssist**

SareptaAssist is a patient support program designed to provide patients with information to help navigate the process of starting and staying on therapy. Sarepta's dedicated team will provide information on insurance benefits, financial assistance options, treatment logistics, options for drug delivery, and ongoing education and support.

MDA's Resource Center provides support, guidance, and resources for patients and families, including information about the approval of Vyondys 53, open clinical trials, and other services. Contact the MDA Resource Center at 1-833-ASK-MDA1 or [ResourceCenter@mdausa.org](mailto:ResourceCenter@mdausa.org).

### **About Vyondys 53**

Vyondys 53 uses Sarepta Therapeutics' exon-skipping technology to target exon 53 of the *DMD* gene. Exon skipping is a treatment strategy in which sections of genetic code are "skipped" (spliced out, or left out) during the protein manufacturing process, allowing cells to create shortened but partially functional dystrophin protein, the muscle protein missing in DMD. Exon skipping is not a cure for DMD but potentially could lessen the severe muscle weakness and atrophy that is the hallmark of the disease.

Just as individuals with DMD caused by a mutation that would be amenable to skipping exon 51 could benefit from treatment with Exondys 51, those with DMD caused by a mutation that would be impacted by skipping exon 53 potentially could benefit from treatment with Vyondys 53.

The FDA's decision to approve Vyondys 53 highlights the importance of years of commitment to supporting and funding breakthrough research by MDA and others into gene identification and unlocking the cause of DMD. MDA-supported research has been central to the development of the exon-skipping approach behind both Exondys 51 and Vyondys 53 from the beginning, having funded foundational work upon which the strategy was built as well as extensive research into the strategy since that time. Laboratory development of exon-skipping therapies began in the 1990s, including notably with MDA-funded work by Steve Wilton, PhD, and colleagues. Their work led to the invention of what would later become Exondys 51 and Vyondys 53.

Since its inception, MDA has committed more than \$218 million to DMD and Becker muscular dystrophy research and more than \$1 billion across the spectrum of neuromuscular diseases.

To learn more about the approval, read the company's [press release](#). For more information about the Essence trial, visit [ClinicalTrials.gov](https://clinicaltrials.gov) and enter "NCT02500381" into the search box.

# EXHIBIT 36

**IN THE UNITED STATES DISTRICT COURT  
DISTRICT OF DELAWARE**

NIPPON SHINYAKU CO., LTD., Plaintiff,	)	
	)	
v.	)	
	)	C.A. No. 21-1015 (MN)
SAREPTA THERAPEUTICS, INC.,	)	
Defendant.	)	
	)	
SAREPTA THERAPEUTICS, INC.,	)	
Defendant and Counter-Plaintiff	)	
	)	
v.	)	
	)	
NIPPON SHINYAKU CO., LTD. and	)	
NS PHARMA, INC., Plaintiff and Counter-	)	
Defendants.	)	

**NIPPON SHINYAKU CO. LTD.’S INITIAL INVALIDITY CONTENTIONS**

Pursuant to Paragraph 7(d) of the of the Proposed Scheduling Order (D.I. 100-1), Plaintiff/Counter-Defendant Nippon Shinyaku Co. Ltd. and Counter-Defendant NS Pharma, Inc. (collectively “NS”) provides the following initial invalidity contentions for each asserted claim, as well as known related invalidating references, to Defendant/Counter-Plaintiff Sarepta Therapeutics, Inc. (“Sarepta”). These Invalidity Contentions disclose the current bases for Defendants’ contentions regarding invalidity, such as prior art references under 35 U.S.C. §§ 102 and 103, lack of written description under 35 U.S.C. § 112, ¶ 1, lack of enablement under 35 U.S.C. § 112 ¶ 1, and indefiniteness under 35 U.S.C. § 112, ¶ 2.<sup>1</sup> These contentions do not address bases for

---

<sup>1</sup> Citations to Title 35 of the United States Code refer to the pre-AIA version of the Code unless otherwise specified below. *See* America Invents Act, P.L. 112-29 (Sept. 16, 2011) at §§ 3(n) and 4(e) (amendments to 35 U.S.C. §§ 102 and 103 take effect and apply to applications filed on or after March 16, 2013, and amendments to 35 U.S.C. § 112 take effect and apply to applications filed on or after September 16, 2012).

unenforceability, including improper inventorship or inequitable conduct.

NS provides this disclosure based on the limited information and evidence available to it at this time, without the benefit of full discovery or the production of Sarepta's documents. NS therefore reserves its right to make any modifications, additions, deletions, or supplementations to this disclosure as additional evidence and information become available, or as is otherwise appropriate and permissible, including any newly discovered prior art or new theories of invalidity.

#### **I. THE UWA PATENTS**

These contentions relate to the claims that Sarepta asserts in its June 17, 2022 Initial Infringement Contentions against NS U.S. Patent Nos. 9,994,851 ("the '851 patent"); 10,227,590 ("the '590 patent"); and 10,266,827 ("the '827 patent") (collectively, "the UWA Patents"). Sarepta's infringement contentions assert claims 1 and 2 of the '851 Patent, claims 1 and 2 of the '590 Patent, and claims 1 and 2 of the '827 Patent (collectively, the "Asserted Claims").

To the extent Sarepta is granted leave to amend its contentions to add any claims from these or other patents, NS will serve supplemental invalidity contentions directed at any such additional claims.

#### **A. Priority**

These invalidity contentions identify prior art based on the priority dates identified by NS's investigation to date:

**Table 1: Priority Dates**

<b>Patent</b>	<b>Priority Date Assessed</b>
'851 Patent	June 28, 2005
'590 Patent	June 28, 2005
'827 Patent	June 28, 2005

**C. INDEFINITENESS**

NS identifies the following claim terms as potentially giving rise to indefiniteness. NS notes that its investigation into terms for construction and the parties exchange of terms for construction may affect these indefiniteness contentions (*e.g.*, resolve these issues and/or highlight additional definiteness issues). As such, NS reserves the right to modify and/or supplement its contentions, including through claim construction disclosures, as it deems appropriate.

<b>Term</b>	<b>Description of Contention</b>
“a target region” <i>’851 Patent, cls. 1, 2; ’590 Patent, cls. 1, 2; ’827 Patent, cl. 1.</i>	This term fails to provide one of skill reasonable certainty regarding the scope of the claim because “target region” is undefined.
“exon 53 of the human dystrophin pre-mRNA” <i>’851 Patent, cls. 1, 2; ’590 Patent, cls. 1, 2; ’827 Patent, cl. 1.</i>	This term fails to provide one of skill reasonable certainty regarding the scope of the claim because “the human dystrophin pre-mRNA” is undefined and lacks antecedent basis.
“wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)” <i>’851 Patent, cls. 1, 2.</i>	This term fails to provide one of skill reasonable certainty regarding the scope of the claim because it is unclear what portion of H53A the “target region” must be within.
“in which uracil bases are thymine bases” <i>’851 Patent, cls. 1, 2; ’590 Patent, cls. 1, 2; ’827 Patent, cl. 1.</i>	This term fails to provide one of skill reasonable certainty regarding the scope of the claim because it is unclear what preceding portion of the claim it is modifying.

Dated: July 18, 2022

MORGAN, LEWIS & BOCKIUS

Amanda S. Williamson (admitted *pro hac vice*)  
Christopher J. Betti (admitted *pro hac vice*)  
Krista V. Venegas (admitted *pro hac vice*)  
Maria E. Doukas (admitted *pro hac vice*)  
Zachary Miller (admitted *pro hac vice*)  
Guylaine Haché (admitted *pro hac vice*)  
Michael T. Sikora (admitted *pro hac vice*)  
110 N. Wacker Drive, Ste 2800  
Chicago, IL 60601  
Telephone: 312.324.1000  
Fax: 312.324.1001  
amanda.williamson@morganlewis.com  
christopher.betti@morganlewis.com  
krista.venegas@morganlewis.com  
maria.doukas@morganlewis.com  
zachary.miller@morganlewis.com  
guylaine.hache@morganlewis.com  
michael.sikora@morganlewis.com

/s/Amy M. Dudash  
Amy M. Dudash (DE Bar No. 5741)  
1201 N. Market Street  
Suite 2201  
Wilmington, Delaware 19801  
Telephone: 302.574.3000  
Fax: 302.574.3001  
amy.dudash@morganlewis.com

*Attorneys for Plaintiff/Counterclaim  
Defendant Nippon Shinyaku Co., Ltd. and  
Counterclaim Defendant NS Pharma, Inc.*

# EXHIBIT 37

**IN THE UNITED STATES DISTRICT COURT  
FOR THE DISTRICT OF DELAWARE**

NIPPON SHINYAKU CO., LTD.,

Plaintiff,

v.

SAREPTA THERAPEUTICS, INC.,

Defendant.

C.A. No. 21-1015 (GBW)

SAREPTA THERAPEUTICS, INC.,

Defendant/Counter-Plaintiff,

v.

NIPPON SHINYAKU CO., LTD.  
and NS PHARMA, INC.

Plaintiff/Counter-Defendants.

**OPENING DECLARATION OF CY A. STEIN, M.D., Ph.D.**

## Table of Contents

	<u>Page</u>
I. INTRODUCTION .....	1
II. QUALIFICATIONS .....	1
III. COMPENSATION AND PRIOR TESTIMONY .....	3
IV. TASK SUMMARY AND MATERIALS CONSIDERED.....	3
V. LEGAL PRINCIPLES .....	4
VI. FACTUAL AND TECHNICAL BACKGROUND.....	5
A. The Wilton Patents.....	5
B. Skilled Artisan .....	7
C. State of the Art .....	8
1. Converting Genetic Information into Proteins.....	8
2. The Pathophysiology of DMD.....	10
3. Exon Skipping Antisense Oligonucleotides as DMD Therapies .....	11
D. Approved Exon Skipping Antisense Oligonucleotides as DMD Therapies .....	14
VII. DISPUTED CLAIM TERMS OF THE WILTON PATENTS.....	15
A. <u>Term 1</u> : “antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA” .....	15
1. A Skilled Artisan Would Have Understood the Meaning of the “Antisense Oligonucleotide” Phrase.....	15
2. The Antisense Oligonucleotide Phrase Is Not Indefinite.....	23
B. <u>Term 1a</u> : “a base sequence”; <u>Term 1b</u> : “a target region”; and <u>Term 1b</u> : “exon 53 of the human dystrophin pre-mRNA” .....	25
C. <u>Term 3</u> : “in which uracil bases are thymine bases” .....	27
1. A Skilled Artisan Would Have Understood that the Claimed “Antisense Oligonucleotide” Has Thymine Bases Instead of Uracil Bases .....	27

2.	The Thymine Bases Clause Is Not Indefinite .....	33
D.	<u>Term 2</u> : “the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)” .....	34
1.	A Skilled Artisan Would Have Understood that the Recited Annealing Sites Identify the Target Region as Within Nucleotides +23 to +69 of Exon 53 of the Human Dystrophin Pre-mRNA .....	34
2.	The Annealing Site Phrase Is Not Indefinite .....	37

I, Cy A. Stein, M.D., Ph.D., declare as follows:

## **I. INTRODUCTION**

1. I have been retained by Sarepta Therapeutics, Inc. (“Sarepta”) as an expert in the above-captioned matter. I understand that Sarepta has asserted claims 1-2 of U.S. Patent No. 9,994,851 (“the ’851 patent”; Ex. 1); claims 1-2 of U.S. Patent No. 10,227,590 (“the ’590 patent”; Ex. 2); and claims 1-2 of U.S. Patent No. 10,266,827 (“the ’827 patent”; Ex. 3) (collectively, the “Wilton patents”) against Nippon Shinyaku Co., Ltd. and NS Pharma, Inc. (collectively, “NS”). As set forth below, I submit this declaration on behalf of Sarepta, offering my opinions on the meaning and scope of certain terms that appear in one or more claims of the Wilton patents and setting forth the basis of my opinions.

## **II. QUALIFICATIONS**

2. I am the Retired Emeritus Arthur and Rosalie Kaplan Professor and Chair of Medical Oncology and Experimental Therapeutics and Retired Emeritus Professor of Molecular and Cellular Biology at the City of Hope Medical Center in Duarte, California. Before I retired, I had practiced medicine, with a specialty in oncology, for over three decades.

3. I received my B.A. in 1974 from Brown University. I received my Ph.D. in Organic Chemistry in 1978 from Stanford University. My doctoral research focused on electron delocalization in ruthenium metal complexes and was supervised by Professor Henry Taube, the winner of the 1983 Nobel Prize in Chemistry.

4. I received my M.D. in 1982 from the Albert Einstein College of Medicine. I completed an internship in Internal Medicine in 1983 and a residency in Internal Medicine in 1985, both at the New York Hospital-Cornell Medical Center. I held a fellowship position between 1985 and 1988 and then a Senior Staff Fellow position between 1988 and 1990 at the National Cancer Institute.

5. In 1990, I became an Assistant Professor of Medicine at Columbia University, and in 1992, I received a second appointment as Assistant Professor of Medicine and Pharmacology. In 1996, I was promoted to Associate Professor of Pharmacology and Medicine, a position I held until 2003. In 2003, I moved to Albert Einstein College of Medicine as Professor of Medicine and Molecular Pharmacology. There, I also held the position of Director of Medical Genitourinary Oncology at the Montefiore Medical Center. In 2012, I moved to City of Hope Comprehensive Cancer Center, appointed as the Interim Deputy Director of Clinical Research. I stayed at City of Hope as a professor and treating physician until I retired in 2020.

6. I have co-authored and published over 140 peer-reviewed articles. Since 1988, I have published about eighty peer-reviewed studies evaluating oligonucleotides as potential therapies for diseases such as cancer, human immunodeficiency virus infection, and restenosis and/or as research tools for investigating biological processes involved in those diseases. To date, I have published about 100 book chapters, review articles, and editorials, the majority of which explore oligonucleotides, including antisense oligonucleotides.

7. In 2004, I co-founded the Oligonucleotide Therapeutics Society, a nonprofit organization that provides a forum for oligonucleotide experts in academia and industry to collaborate and advance the research and development of oligonucleotides as therapeutics. I served as Treasurer of the Oligonucleotide Therapeutics Society from 2004 to 2008 and also served in an ex officio capacity in 2009. I was awarded the Lifetime Achievement Award by the organization in 2022 in recognition of my lasting commitment to the field of oligonucleotide therapeutics and contributions to education, research, and therapeutic applications.

8. I have served on editorial boards for many years, including as the Co-Editor-in-Chief of *Oligonucleotides* (from 1993 to 2014; formerly known as *Antisense and Nucleic Acid*

*Drug Development and Antisense Research and Development*), the Series Editor for *Perspectives in Antisense Science*; and the Deputy Editor for *Molecular Therapy-Nucleic Acids* (from 2016 to 2019). In addition, I have served on various editorial advisory boards, including as the Associate Editor for *Clinical Cancer Research* and Deputy Editor of *Molecular Therapy-Oncolytics*.

9. My work has been extensively recognized by my professional community. In addition to the Lifetime Achievement Award from the Oligonucleotide Therapeutics Society, I have been recognized as one of the “Top Doctors” (Oncology) in Los Angeles (*Hollywood Reporter*, 2014 and 2015) and the Los Angeles Area (*Pasadena Magazine*, 2013, 2014, and 2015), with similar recognitions in the New York area (*The New York Times*, 2008 and 2009; *New York Magazine*, 2008 and 2009). My research papers have been highlighted for their quality and impact factor by F1000 (2009), Hidden Jewels (2009), the Oligonucleotide Therapeutics Society (2016), and others.

10. I am an inventor of several issued U.S. patents, including those that involve uses of oligonucleotides. My professional qualifications are described in further detail in my curriculum vitae, which is attached as **Appendix A**.

### **III. COMPENSATION AND PRIOR TESTIMONY**

11. I am being compensated for the time I spend on this matter at a rate of \$600 per hour. My compensation is not contingent upon my opinions offered herein or the outcome of this matter. Within the last four years, I have testified as an expert in *In re: E. I. Du Pont de Nemours and Company C-8 Personal Injury Litigation*, 2:13-MD-02433 (S.D. Ohio).

### **IV. TASK SUMMARY AND MATERIALS CONSIDERED**

12. I have been asked to offer opinions on the meaning and scope of the following terms, assessed from the perspective of a skilled artisan at the time when the Wilton patents were filed, i.e., June 28, 2005:

Term 1: “antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA”;

Term 1a: “a base sequence”;

Term 1b: “a target region”;

Term 1c: “exon 53 of the human dystrophin pre-mRNA”;

Term 2: “the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)”;

Term 3: “in which uracil bases are thymine bases.”

13. In forming my opinions, I have considered the claim language, specification, and select prosecution histories of the Wilton patents, together with publications and knowledge common in the art in 2005. In addition, I considered the proposed constructions and contentions set forth by Sarepta and NS to date. I have also considered the materials cited in this declaration, as well as those listed in **Appendix B**. To the extent I am provided with additional documents or information, including any expert declaration(s) in this case, I reserve the right to modify, supplement, and/or expand my opinions based on this information. I may also consider additional documents and information in forming any supplemental opinions. I reserve the right to prepare demonstrative exhibits to assist in illustrating my opinions at the claim construction hearing.

## **V. LEGAL PRINCIPLES**

14. I am not a legal expert and therefore offer no legal opinions. Instead, counsel for Sarepta has explained certain legal principles to me, which I have applied in providing my opinions.

15. I have been informed that a claim term of a patent must be interpreted from the perspective of a skilled artisan in the pertinent art at the time of the invention. I understand that

the interpretation is primarily based on the intrinsic evidence, which consists of the patent claims, specification, and prosecution history. I further understand that a claim term is given its plain and ordinary meaning, except when the specification provides a specific definition or when the specification or the prosecution history reveals that the inventor applied a different meaning than it would otherwise have to a skilled artisan.

16. In addition to intrinsic evidence, I understand that extrinsic evidence, e.g., scientific publications, treatises, and dictionaries, may be considered.

17. I have been informed that a claim satisfies the definiteness requirement when it informs a skilled artisan, with reasonable certainty, of the meaning and scope of the claimed invention. I understand that the definiteness inquiry must be carried out from the perspective of a skilled artisan at the time of the invention, reading the claim in light of the patent's specification and prosecution history.

## **VI. FACTUAL AND TECHNICAL BACKGROUND**

### **A. The Wilton Patents**

18. The Wilton patents generally describe antisense oligonucleotides that can cause skipping of various exons in the human dystrophin pre-mRNA including exon 53 and their use for treating Duchenne Muscular Dystrophy ("DMD"). From my review, the specifications of the Wilton patents do not substantially differ from each other. Thus, I will refer to the specification of the '851 patent as representative of the specification of all three Wilton patents, unless otherwise indicated.

19. Claim 1 of the '851 patent recites:

1. An antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA,

wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69), wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, and wherein the antisense oligonucleotide induces exon 53 skipping; or a pharmaceutically acceptable salt thereof.

20. Claim 2 of the '851 patent is directed to a pharmaceutical composition comprising a pharmaceutically acceptable carrier and an antisense oligonucleotide having the characteristics recited in claim 1 of the '851 patent.

21. Claim 1 of the '590 patent is similar to claim 1 of the '851 patent but does not recite that the claimed target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69):

1. An antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA, wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, and wherein the antisense oligonucleotide induces exon 53 skipping; or a pharmaceutically acceptable salt thereof.

22. Claim 2 of the '590 patent is directed to a pharmaceutical composition comprising a pharmaceutically acceptable carrier and an antisense oligonucleotide having the characteristics recited in claim 1 of the '590 patent.

23. Claim 1 of the '827 patent recites:

1. A method for treating a patient with Duchenne muscular dystrophy (DMD) in need thereof who has a mutation of the DMD gene that is amenable to exon 53 skipping, comprising administering to the patient an antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA, wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, and wherein the antisense oligonucleotide induces exon 53 skipping; or a pharmaceutically acceptable salt thereof.

24. Claim 2 of the '827 patent depends from claim 1 of the '827 patent and further recites that the claimed antisense oligonucleotide is administered intravenously.

**B. Skilled Artisan**

25. For the purpose of this declaration, I have been asked to assume that the Wilton patents were filed on June 28, 2005. Unless indicated otherwise, my opinions regarding a skilled artisan apply to such a person as of June 28, 2005.

26. In my opinion, a skilled artisan with respect to the claimed invention of the Wilton patents would have had a Ph.D. in chemistry, biochemistry, cell biology, genetics, molecular biology, or an equivalent, and several years of experience with antisense oligonucleotides for inducing exon skipping. A skilled artisan also would have been familiar with methods for making and testing the safety and efficacy of antisense oligonucleotides, both *in vitro* and *in vivo*, and the use of antisense oligonucleotides for inducing exon skipping in the context of medical conditions, such as DMD, that may be treated by administering antisense oligonucleotides. A skilled artisan

would have had knowledge of and experience with chemical modifications that may be incorporated into antisense oligonucleotides, such as modifications to the backbone and/or nucleobases of the antisense oligonucleotides, and the potential impact of those modifications on the utility of the antisense oligonucleotides.<sup>1</sup>

### **C. State of the Art**

#### **1. Converting Genetic Information into Proteins**

27. Deoxyribonucleic acid (“DNA”) provides the blueprint for every protein in the body. Ex. 7 (Alberts 2002) at 191–92. DNA molecules contain two complementary strands, each of which is a long chain composed of four different kinds of subunits (referred to as “nucleotides”). *Id.* at 193. Each different nucleotide has a different nucleobase (or simply, “base”). *Id.* In DNA, there are four types of bases: adenine (A), guanine (G), cytosine (C), and thymine (T). *Id.* The linear string of nucleotides and their bases in a DNA strand provides coded instructions by the base order (also referred to as a “base sequence”) for producing all proteins in an organism. *Id.* at 299.

28. The nucleotides in each string are linked to one another via linkages between the 5’ phosphate group of one nucleotide and the 3’ hydroxyl group of another nucleotide. *Id.* at 193. Thus, the base sequence of a DNA molecule is written in a specific direction, starting from the 5’-end and ending at the 3’-end. *Id.* A segment of the DNA sequence that encodes a particular protein

---

<sup>1</sup> I understand that NS has asserted seven patents against Sarepta in this case, all of which are assigned to Nippon Shinyaku Co., Ltd. and National Center of Neurology and Psychiatry. I further understand that Sarepta challenged these patents in proceedings at the Patent Office and in those proceedings, Sarepta proposed the same definition of a skilled artisan. Because I agree with the definition, I have adopted it in rendering my opinions here.

is a “gene.” *Id.* at 299, 301. Converting genetic information into a protein involves three main steps: transcription, splicing, and translation. *Id.* at 301, 315–16. Each step is explained below.

29. Transcription: To generate proteins, the sequence of bases in the DNA is first copied (“transcribed”) into a molecule known as ribonucleic acid (“RNA”). *Id.* at 302. Like DNA, RNA is a linear chain of nucleotides. *Id.* RNA nucleotides have the same bases as DNA, except RNA has uracils (U) instead of thymines (T). *Id.* Genes contain regions that are ultimately expressed in the relevant protein (“exons”) interspersed with regions that are not (“introns”). *Id.* at 315–17. The transcription machinery copies the entire gene sequence, including both exons and introns, and produces a molecule known as precursor messenger RNA (“pre-mRNA”). *Id.* at 302, 315–17.

30. Splicing: Introns must be removed from the pre-mRNA through a process called “splicing” before the protein synthesis machinery can interpret it to produce a functional protein. *Id.* at 315–17. Splicing involves specialized groups of proteins (“spliceosomes”) that bind to the beginning and end of each intron (referred to as the “donor” and “acceptor” regions, respectively), excise the intron, and connect the remaining exons together. *Id.* at 319–20; *see also* Ex. 19 (Mann 2002) at 646. Through splicing, the pre-mRNA is converted into messenger RNA (“mRNA”). Ex. 7 (Alberts 2002) at 304, 317.

31. Translation: Once the mRNA is generated, its sequence of bases is decoded to produce a protein via a process called translation. *Id.* at 336. During this process, the translation machinery parses the mRNA base sequence in groups of three bases, or “codons,” to decode the protein sequence. *Id.* Each possible sequence of three bases translates into one of twenty possible amino acids or into a “stop codon,” the latter of which signals the completion of the protein’s amino acid sequence. *Id.* The translation machinery reads these codons and assembles

corresponding amino acids as a sequence of amino acids. *Id.* at 344–46. The sequence of amino acid acids then fold into a protein that carries out biological functions in the body. *Id.* at 350, 354–55.

32. The mRNA sequence must be parsed correctly to produce the proper sequence of amino acids in the protein. *Id.* at 336, 348–49. In other words, it is vital that the mRNA is read in the correct frame (e.g., starting with the first nucleotide and not with the second or third). *Id.* However, if a genetic mutation results in disruption of the reading frame (e.g., by the deletion of a nucleotide), the remainder of the codons will not be read correctly. *Id.* Mutations that disrupt the reading frame thus affect the ultimate sequence of amino acids, which can cause a partial or total loss of the protein’s expression and/or function. *Id.* at 348–49.

## **2. The Pathophysiology of DMD**

33. DMD is a devastating genetic disorder that affects one in about 3,500 newborn males and causes ultimately fatal muscular defects. Ex. 19 (Mann 2002) at 644–45; Ex. 30 (van Deutekom 2001) at 1547; Ex. 32 (Wilton 1999) at 334. Affected individuals progressively lose their ability to walk and develop respiratory and cardiac complications. Ex. 19 (Mann 2002) at 644–45; Ex. 30 (van Deutekom 2001) at 1547; Ex. 8 (Botvin Madorsky 1984) at 79. Without treatment, the life expectancy of affected individuals is less than 30 years. Ex. 8 (Botvin Madorsky 1984) at 79; Ex. 16 (Hoffman 1987) at 347.

34. DMD occurs due to the body’s inability to synthesize dystrophin protein, which is important to the functioning of muscle fibers. Ex. 19 (Mann 2002) at 644–45; Ex. 30 (van Deutekom 2001) at 1547. Dystrophin protein is encoded by the dystrophin gene, which contains 79 exons. Ex. 32 (Wilton 1999) at 330–31. The dystrophin gene was identified in the mid-1980s, and the base sequence of each exon of the gene was deciphered shortly thereafter. Ex. 16 (Hoffman 1987) at Abstract; Ex. 18 (Koenig 1988) at Abstract.

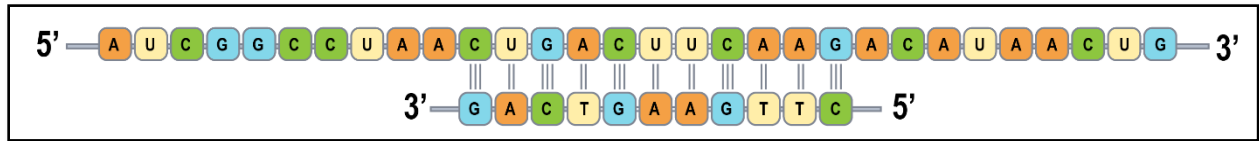
35. The lack of dystrophin in DMD patients is caused by mutations in the dystrophin gene that disrupt the reading frame of the dystrophin mRNA. Ex. 32 (Wilton 1999) at 330. This disruption causes premature termination of dystrophin synthesis and a lack of functional dystrophin protein in the body. *Id.*

### **3. Exon Skipping Antisense Oligonucleotides as DMD Therapies**

36. In the 1990s, researchers began investigating the concept of “exon skipping” as an approach to treating DMD. Ex. 32 (Wilton 1999) at 335. Therapies based on this concept aimed to excise, i.e., “skip,” the targeted exon from the pre-mRNA during the splicing process. Ex. 19 (Mann 2002) at 645; Ex. 30 (van Deutekom 2001) at 1547; Ex. 32 (Wilton 1999) at 331. By excluding the targeted exon from the final mRNA transcript, exon skipping sought to restore the correct reading frame, resulting in the production of functional dystrophin. Ex. 30 (van Deutekom 2001) at 1547. Researchers hypothesized that while dystrophin protein translated from the resulting mRNA would be shorter than its wild-type (normal) counterpart, the resulting protein could still exhibit functional properties necessary for the health of muscle fibers in DMD patients. Ex. 30 (van Deutekom 2001) at 1548; Ex. 32 (Wilton 1999) at Abstract, 334–35.

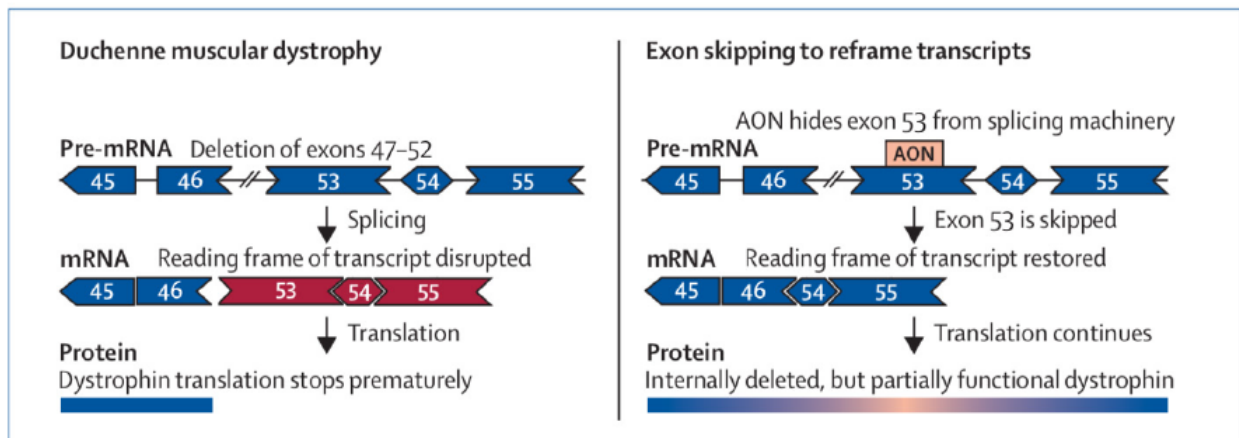
37. Around the same time, researchers began exploring antisense oligonucleotides as a therapy for achieving exon skipping in cells. Ex. 19 (Mann 2002) at 645; Ex. 30 (van Deutekom 2001) at 1547; Ex. 32 (Wilton 1999) at 331. An antisense oligonucleotide is a short chain of nucleotides designed to target a specific portion of the pre-mRNA (often referred to as a “target region”) by having a base sequence that is complementary to the target region. Ex. 19 (Mann 2002) at 645, 649; Ex. 24 (Summerton 1997(a)) at 187. The bases of an antisense oligonucleotide are “complementary” to the bases in that portion of the transcript, i.e., adenine (A) pairs with thymine (T) (or uracil (U)), and cytosine (C) pairs with guanine (G). Ex. 7 (Alberts 2002) at 192–95, 302–03; Ex. 24 (Summerton 1997(a)) at 187. This pairing between the complementary bases

is referred to as Watson-Crick base pairing. *See* Ex. 7 (Alberts 2002) at 192–95. **Figure 1** below illustrates the “complementarity” of two sequences, each comprising a unique set of nucleobases in a particular order. The sequences of two strands are “complementary” because the bases in one strand arranged in one direction can pair with their counterpart bases in another strand arranged in a reverse direction:



**Figure 1.** Complementary Base Sequences

38. In the case of “exon skipping,” an antisense oligonucleotide is designed to interact with the pre-mRNA molecule and interfere with the splicing process—such that the targeted exon is excluded from the mRNA transcript. Ex. 19 (Mann 2002) at 645; Ex. 30 (van Deutekom 2001) at 1547; Ex. 32 (Wilton 1999) at 331. **Figure 2** is a schematic drawing illustrating exon skipping caused by an antisense oligonucleotide.

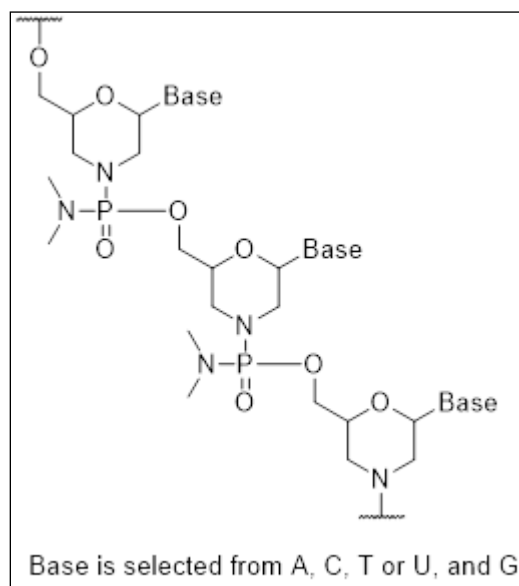


**Figure 2.** Exon Skipping Antisense Oligonucleotide  
(Adopted from Ex. 34 (Aartsma-Rus 2009) at 873 (Fig. 1))

39. Without chemical modification, antisense oligonucleotides made of naturally occurring nucleotides rapidly degrade in the body. Ex. 25 (Summerton 1997(b)) at 63. To

minimize degradation, chemically modified nucleotide analogs are used as building blocks. Ex. 24 (Summerton 1997(a)) at 187–88; Ex. 25 (Summerton 1997(b)) at 63. Like their natural counterparts, these analogs contain bases that are capable of interacting with the pre-mRNA. Ex. 24 (Summerton 1997(a)) at 187–89. But these nucleotide analogs are more resistant to degradation, and therefore are more likely to exert desired therapeutic effects. Ex. 26 (Summerton 1999) at 142.

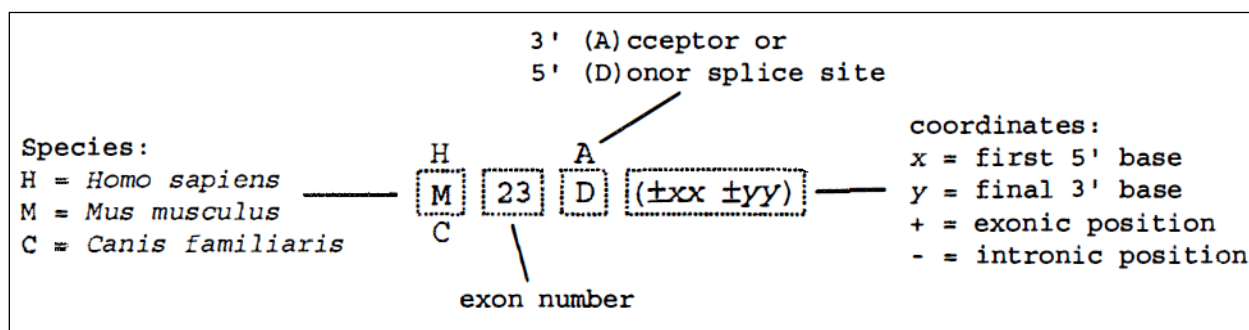
40. One such modification is known as a “morpholino.” Ex. 24 (Summerton 1997(a)) at 188–89. The chemical structure of a morpholino oligomer is depicted in **Figure 3**.



**Figure 3.** A Chemically Modified “Morpholino” Oligomer (adapted from Ex. 24 (Summerton 1997(a)) at 188 (Fig. 2))

41. By 2005, scientists had developed a nomenclature system for exon skipping antisense oligonucleotides directed to the dystrophin gene. Ex. 19 (Mann 2002) at 644–46. As shown in **Figure 4**, the system consisted of four identifiers collectively defining the region of the pre-mRNA targeted by an antisense oligonucleotide: **Letter / Number / A or D / Coordinates**. *Id.* at 646 (Fig. 1(A)). Here, “**Letter**” corresponds to the single letter designation of the species, “**Number**” refers to the number of the exon targeted by the antisense oligonucleotide, “**A or D**”

signifies the origin point (i.e., an acceptor site at the beginning present at the '5-end of the exon or a donor site at the end of the exon present the '3-end of the exon), and “**Coordinates**” designate the beginning and end of the target region by the number of bases from the origin. *Id.* In addition, each coordinate number indicates whether that coordinate occurs in the intron (“-”) or the exon (“+”). *Id.* For example, “H53A(+36+60)” corresponds to nucleotides +36 to +60 (signified by “(+36+60)”) counted from the beginning (signified by “A”) of exon 53 (signified by “53”) of the human dystrophin pre-mRNA (signified by “H”).



**Figure 4.** Nomenclature System for Exon Skipping Antisense Oligonucleotides  
(Adapted from Ex. 19 (Mann 2002) at 626 (Fig. 1(A)))

#### **D. Approved Exon Skipping Antisense Oligonucleotides as DMD Therapies**

42. In 2016, Exondys 51<sup>®</sup> (eteplirsen), developed by Sarepta, became the first DMD drug approved by the Food and Drug Administration (“FDA”). Ex. 12 (FDA 2016). Exondys 51<sup>®</sup> (eteplirsen) is an exon skipping antisense oligonucleotide indicated for treating DMD patients who are amenable to exon 51 skipping. Ex. 9 (Eteplirsen Label).

43. In 2019, Sarepta received FDA approval for another exon skipping antisense oligonucleotide, Vyondys 53<sup>®</sup> (golodirsen), indicated for treating DMD patients who are amenable to exon 53 skipping. Ex. 13 (FDA 2019); Ex. 15 (Golodirsen Label). Vyondys 53<sup>®</sup> (golodirsen) was the second DMD exon skipping drug approved by FDA. Ex. 35 (MDA 2019). Later, in 2020, NS obtained FDA approval for its exon skipping antisense oligonucleotide known as Viltepso<sup>®</sup>

(viltolarsen), which is also indicated for treating DMD patients amenable to exon 53 skipping. Ex. 14 (FDA 2020); Ex. 31 (Viltolarsen Label).

## VII. DISPUTED CLAIM TERMS OF THE WILTON PATENTS

- A. **Term 1:** “antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA”

Sarepta’s Proposed Construction	NS’s Proposed Construction
<p><i>Not indefinite</i></p> <p><i>No construction needed in light of inter alia, the intrinsic evidence and the knowledge of a person of ordinary skill in the art</i></p> <p>To the extent construction is needed, Sarepta proposes that the phrase should be given its plain and ordinary meaning, i.e.,</p> <p>“antisense oligonucleotide that has 20 to 31 bases, which collectively form a sequence that is 100% complementary to a segment of the pre-mRNA transcribed from exon 53 of the human dystrophin gene”</p>	<p><i>Indefinite</i></p> <p>Or, in the alternative:</p> <p>“antisense oligonucleotide with 20 to 31 bases that includes any sequence of bases that is part of the antisense oligonucleotide that are 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA”</p>

### 1. A Skilled Artisan Would Have Understood the Meaning of the “Antisense Oligonucleotide” Phrase

44. The phrase “antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA” (referred to here as the “antisense oligonucleotide” phrase) appears in each claim of the Wilton patents. *See supra* ¶¶18–24.

45. I understand that Sarepta proposes considering this phrase in its entirety, whereas NS proposes excerpting out three subparts within the phrase (“a base sequence,” “a target region,” and “exon 53 of the human dystrophin pre-mRNA”) and analyzing each separately. In my opinion, Sarepta’s proposal should be adopted because reading the phrase as a whole provides the

appropriate context for those three subparts. And reading the phrase as a whole, a skilled artisan would have understood it to mean an antisense oligonucleotide that has 20 to 30 bases, which collectively form a sequence (“base sequence”) that is 100% complementary to a segment of the pre-mRNA (“target region”) transcribed from exon 53 of the human dystrophin gene (“exon 53 of the human dystrophin pre-mRNA”).

**a. The Specification of the Wilton Patents**

46. Using the terminology that appears in the antisense oligonucleotide phrase, the specification of the Wilton patents explains what an antisense oligonucleotide is, in particular an antisense oligonucleotide used to induce exon skipping. Below I have summarized exemplary discussions in the specification that inform the meaning of the antisense oligonucleotide phrase and terms within it.

47. “pre-mRNA”: The specification explains that in various genetic diseases, “the effects of mutations on the eventual expression of a gene can be modulated through a process of targeted exon skipping during the splicing process.” Ex. 1 (the ’851 patent) at 2:20–23. The specification explains that this splicing process is directed by biological machinery that interacts with pre-mRNA transcribed from the gene of interest. *Id.* at 2:23–32. As the specification further explains, “[b]y changing the way the splicing machinery reads or recognises the motifs involved in pre-mRNA processing, it is possible to create differentially spliced mRNA molecules.” *Id.* at 2:32–35.

48. “target region” in the “human dystrophin pre-mRNA”: The specification explains that an antisense oligonucleotide may be used to achieve targeted exon skipping and identifies the human dystrophin gene as a potential candidate. *Id.* at 2:38–41, 2:55–63, 24:61–25:6. For the human dystrophin gene specifically, the specification explains that antisense oligonucleotides are “capable of binding to specified *dystrophin pre-mRNA targets* and re-directing processing of that

gene.” *Id.* at 23:38–41; *see also id.* at 23:43–45 (antisense oligonucleotides “capable of binding to a selected *target*”), 24:61–25:11 (antisense oligonucleotides are directed to “*a selected target in the dystrophin pre-mRNA*”), 24:48–60 (“target sites” can identified based on a “gene” or the “mRNA transcribed [from] the gene”). Through these disclosures, the specification informs a skilled artisan that the antisense oligonucleotide of interest is directed to a segment within the pre-mRNA (“target region”), which is transcribed from the human dystrophin gene (“the human dystrophin pre-mRNA”).

49. “exon 53 of the human dystrophin pre-mRNA”: As the specification explains, in some cases, this “target” may be present at various places in the human dystrophin pre-mRNA, including, e.g., within an exon of the human dystrophin pre-mRNA. *Id.* at 23:24–27 (antisense oligonucleotides “targeted to nucleotide sequences . . . in exons within pre-mRNA sequences”); *see also id.* at 24:38–47 (exon skipping induced by targeting “intra-exonic regions”). Among 79 exons of the human dystrophin gene and its corresponding transcribed pre-mRNA, the specification identifies exon 53 as a candidate exon (“exon 53 of the human dystrophin pre-mRNA”). *Id.* at 25:2–11, 4:47–49.

50. “base sequence” “complementary” to the “target region”: As the specification also explains, an antisense oligonucleotide is capable of recognizing and interacting with its target region because they have complementary “sequences.” *Id.* at 25:18–38. As discussed above, the sequences of two nucleotide strands are complementary when the bases in one strand arranged in one direction can pair with their corresponding bases in another strand, arranged in a reverse direction. *See supra* ¶37. Here, the sequence of the target region is present in the pre-mRNA and is made of four bases, adenine (A), uracil (U), cytosine (C), and guanine (G), arranged in a linear order. The sequence of the antisense oligonucleotide is similarly made of bases, which are also

arranged in a linear order (“base sequence”) and are capable of pairing with the bases in the target region. Exemplary antisense oligonucleotides in the specification illustrate this complementarity concept. As reproduced below, Table 1A of the specification describes exemplary antisense oligonucleotides directed to various exons of the human dystrophin pre-mRNA. Each antisense oligonucleotide is defined by two types of information: (1) its base sequence made of adenine (A), uracil (U) (or thymine (T), if it is for a “morpholino”), cytosine (C), and guanine (G) (“NUCLEOTIDE SEQUENCE”) and (2) the complementary target region within the human dystrophin pre-mRNA (“SEQUENCE”).

TABLE 1A		
Description of 2'-O-methyl phosphorothioate antisense oligonucleotides that have been used to date to study induced exon skipping during the processing of the dystrophin pre-mRNA. Since these 2'-O-methyl antisense oligonucleotides are more RNA-like, U represents uracil. With other antisense chemistries such as peptide nucleic acids or morpholinos, these U bases may be shown as "T".		
SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5'-3')
1	H8A(-06+18)	GAU AGG UGG UAU CAA CAU CUG UAA
2	H8A (-03+18)	GAU AGG UGG UAU CAA CAU CUG
3	H8A(-07+18)	GAU AGG UGG UAU CAA CAU CUG UAA G
4	H8A(-06+14)	GGU GGU AUC AAC AUC UGU AA
5	H8A(-10+10)	GUA UCA ACA UCU GUA AGC AC

**Figure 5.** Excerpt of Table 1A of the '851 Patent

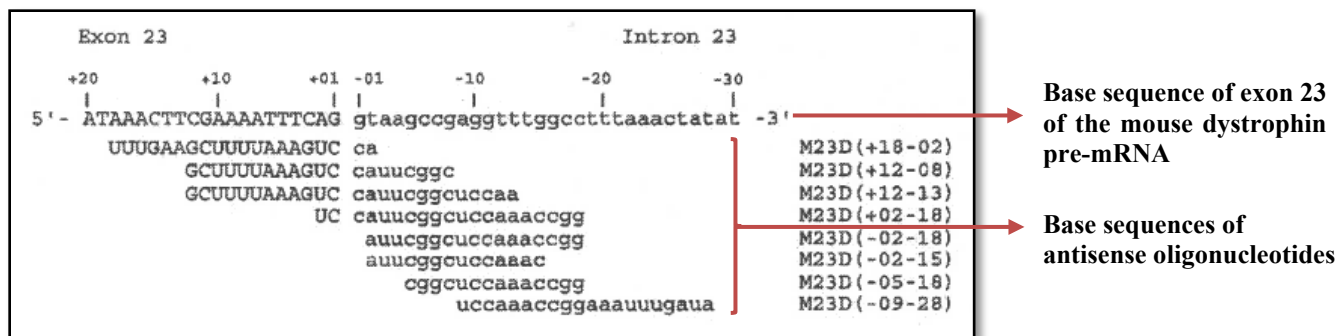
51. The specification indicates that the entire linear sequence of bases in an antisense oligonucleotide forms a base sequence, i.e., the overall length of an antisense oligonucleotide is the same as the length of the base sequence itself. For example, the specification defines the length of an antisense oligonucleotide based on the number of bases contained in the antisense

oligonucleotide. Ex. 1 (the '851 patent) at 23:62–24:3, 25:61–26:3. The specification refers to those bases as a “sequence[.]” *Id.*

52. A skilled artisan reading the specification would have understood that a linear sequence of the bases in an antisense oligonucleotide (“base sequence”) is complementary to a segment in the human dystrophin pre-mRNA (“target region”), in particular the pre-mRNA transcribed from exon 53 of the human dystrophin gene (“exon 53 of the human dystrophin pre-mRNA”) for the Wilton patents.

**b. The Usage of the Claim Terms in the Art**

53. The specification’s use of the claim terms is consistent with how those terms are used and understood in the art. For example, the inventors of the Wilton patents co-authored an article in 2002, evaluating several antisense oligonucleotides directed to exon 23 of the mouse dystrophin pre-mRNA. Ex. 19 (Mann 2002) at Abstract. The inventors explained that antisense oligonucleotides had potential for treating DMD because they may be able to “induce *targeted* removal of disease-causing *exons from pre-mRNA transcripts* during splicing.” *Id.* at 645 (emphasis added). Using mouse cells as a model system, the inventors tested several antisense oligonucleotides and identified an “exon 23 *target site*” amenable to exon skipping—the term that the inventors used to define the “exact annealing position” relative to the target exon. *Id.* at Abstract, 646 (emphasis added). The inventors’ illustration, reproduced below, shows perfect complementarity between the base sequences of the dystrophin pre-mRNA and the tested antisense oligonucleotides:



**Figure 6.** Annotated Figure 1B Adopted from Ex. 19 (Mann 2002) at 646

54. Another article published in 2002 by independent researchers used the same terminology to describe exon skipping antisense oligonucleotides. Ex. 4 (Aartsma-Rus 2002) at S71–S72. In this article, researchers from Leiden University stated that antisense oligonucleotides “can bind to *specific sequences within the DMD pre-mRNA*,” for example, “*target[]sequences* involved in the splicing process.” *Id.* at S71 (emphasis added). These researchers explored antisense oligonucleotides “designed to bind to exon-internal *target sequences*” in the human dystrophin “*pre-mRNA*.” *Id.* at S72 (emphasis added). The base sequences of the tested antisense oligonucleotides are reported in Table 1 of the article, a portion of which is reproduced below:

Name	Antisense sequence (5'–3')	Length (bp)	G/C%	U/C%	Exon skip	Transcript
h2AON 1	cccauuuugugaauuuuuuuuu	24	29	75	+	OF
h2AON 2	uugugcauuuacccaauuugug	22	36	68	–	OF
h29AON 1	uauccucugaauugcgauc	20	45			IF
h29AON 2	gguauccucugaauugcgc	20	50			IF
h40AON 1	gagccuuuuuuucuuuug	19	37			IF
h40AON 2	uccuuucgucucugggcuc	19	58	79	+	IF
h41AON 1	cuccucuuuuucuuucugc	19	47	95	+	IF

Base sequences of antisense oligonucleotides

**Figure 7.** Annotated Table 1 Adopted from Ex. 4 (Aartsma-Rus 2002) at S73

55. Several contemporaneous review articles also support the common usage of the terminology that appears in the claims of the Wilton patents. *See, e.g.*, Ex. 33 (Wilton 2005) at 225 (2005 review article from the inventors of the Wilton patents summarizing studies that demonstrated “the exquisite sensitivity of [antisense oligonucleotides] in *targeting specific sites*”

in differentiating the human versus murine “*dystrophin pre-mRNA*”); Ex. 5 (Aartsma-Rus 2007) at 1620 (2007 review article from researchers at Leiden University explaining that “exon skipping strategy *targets the pre-mRNA transcribed from the endogenous gene*”) (emphasis added).

56. A review article from Dr. Takeda illustrates the same. I understand that Dr. Takeda is a named inventor of the NS patents asserted by NS against Sarepta in this case. *See* Ex. 29 (U.S. Patent No. 9,708,361) at item (72). In 2007, he co-authored a review article summarizing potential DMD therapies, one of which involved the use of exon skipping antisense oligonucleotides. Ex. 28 (Suzuki 2007) at Abstract, 92. Dr. Takeda explained that “[e]xon skipping using *antisense oligonucleotides (AOs) targets transcribed RNA molecules* to omit a nonsense mutation and restore a disrupted reading frame.” *Id.* at 94 (emphasis added). Dr. Takeda’s explanation of the antisense oligonucleotide technology and use of terms such as “target” and “transcribed RNA” is consistent with my proposed construction of the antisense oligonucleotide phrase.

57. Notably, the U.S. Patent and Trademark Office (“Patent Office”) also used the same terminology in a consistent manner in describing similar subject matter, further highlighting the wide acceptance of the terminology in the art. I understand that a patent related to the Wilton patents was previously involved in a Patent Office proceeding. After considering contemporaneous evidence submitted by the parties involved, the Patent Office summarized the subject matter at issue as “an *oligonucleotide* that includes *a nucleobase sequence* that is complementary to *a portion of a particular pre-mRNA exon*,” in particular “*exon 53 of the pre-mRNA* associated with the gene responsible for the formation of the protein dystrophin.” Ex. 17 (Interference No. 106,007 Decision) at 3 (emphasis added).

58. In sum, a skilled artisan reading the “antisense oligonucleotide” phrase would have understood what it means. The specification uses the phrase and its terms to explain antisense

oligonucleotide technology in the context of inducing exon skipping of the human dystrophin pre-mRNA. Indeed, the specification's usage is consistent with the way numerous contemporaneous articles described the same technology. As such, I do not believe that a specific construction is necessary for this phrase.

**c. Sarepta's and NS's Proposed Constructions**

59. To the extent that a construction of the antisense oligonucleotide phrase is necessary, my opinion is that it should be given its plain and ordinary meaning. Sarepta's construction conveys this meaning, i.e., an antisense oligonucleotide that has 20 to 31 bases, which collectively form a sequence ("base sequence") that is 100% complementary to a segment of the pre-mRNA ("target region") transcribed from exon 53 of the human dystrophin gene ("exon 53 of the human dystrophin pre-mRNA").

60. In contrast, I understand that NS proposes construing three separate terms, "base sequence," "target region," and "exon 53 of the human dystrophin pre-mRNA." In my opinion, this is illogical. As illustrated above, these terms are interdependent since they are part of the antisense oligonucleotide phrase. Indeed, an "antisense oligonucleotide" can be properly understood only if it is considered in the context of its "base sequence." The "base sequence" in turn can only be properly understood when considered in the context of its complementarity to its "target region." The "target region" in turn can only be properly understood when considered in the context of where it appears, in this case, in "exon 53 of the human dystrophin pre-mRNA." These terms should also be considered in the context of antisense oligonucleotide technology and how skilled artisans had been using them for years before the Wilton patents were filed. Considering these highly interconnected technical terms out of context, as NS proposes, deprives them of their scientific meaning.

61. NS's alternative construction of the antisense oligonucleotide phrase as "antisense oligonucleotide with 20 to 31 bases that includes any sequence of bases that is part of the antisense oligonucleotide that are 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA" does not cure this deficiency. NS's construction simply repeats the antisense oligonucleotide phrase while swapping out the term "base sequence" with "any sequence of bases that is part of the antisense oligonucleotide." I disagree with NS's construction for at least two reasons. First, for the reasons discussed below, I disagree that the term "base sequence" means "any" sequence of bases that is "part" of the antisense oligonucleotide. *See infra* ¶¶68–69. Second, in simply repeating the remainder of the phrase, NS's construction does not explain what the phrase means in plain language.

62. For at least these reasons, my opinion is that Sarepta's construction, which accurately explains what an antisense oligonucleotide is, should be adopted.

## **2. The Antisense Oligonucleotide Phrase Is Not Indefinite**

63. I understand that NS argues that the antisense oligonucleotide phrase is indefinite. I further understand that NS has made two arguments: (1) the term "target region" that appears within the phrase is purportedly indefinite because it is not expressly defined in the specification and (2) the term "exon 53 of the human dystrophin pre-mRNA" that also appears within the phrase is purportedly indefinite because it is not expressly defined in the specification and refers to "the" human dystrophin pre-mRNA without first introducing "a" human dystrophin pre-mRNA. I disagree with both of NS's arguments.

64. As discussed above, a skilled artisan would have understood, with reasonable certainty, what the antisense oligonucleotide phrase means. *See supra* ¶¶46–58. The terms within the phrase such as "target region" and "exon 53 of the human dystrophin pre-mRNA" had been commonly and widely used in the art for many years to describe exon skipping antisense

oligonucleotides. *See supra* ¶¶53–58. Consistent with the common usage of these terms in the art, the specification repeatedly uses the phrase and its terms to explain exon skipping antisense oligonucleotides in the context of DMD treatments. *See supra* ¶¶46–52. There was no need for the specification to expressly define these technical terms, which would have been understood by a skilled artisan.

65. NS’s indefiniteness challenge to the claim language “exon 53 of the human dystrophin pre-mRNA” makes no sense in view of the state of the art. The dystrophin gene was discovered nearly twenty years before the Wilton patents were filed. *See supra* ¶34. The sequence of each exon within the dystrophin gene was well known when the Wilton patents were filed. *See supra* ¶34; *see* Ex. 18 (Koenig 1988) at Summary (“The complete sequence of the human Duchenne muscular dystrophy cDNA has been determined.”); *see id.* at 220–21 (Fig. 1) (“Nucleotide Sequence of the DMD Transcript and Deduced Primary Structure of the Encoded Protein”). A skilled artisan would have understood the precise meaning of “exon 53 of the human dystrophin pre-mRNA.” In fact, since there is only one sequence, i.e., “the” sequence, known in the art for exon 53 of the human dystrophin pre-mRNA, the use of “the” instead of “a” in this context makes sense and would not have been unclear to a skilled artisan.

66. NS’s U.S. Patent No. 9,708,361 (“the ’361 patent”) and its prosecution history further demonstrate that outside of this case, NS understood the meaning of the terminology used in the antisense oligonucleotide phrase. During prosecution of the ’361 patent, the Examiner raised an obviousness rejection, contending that a prior art reference “taught antisense based alteration of splicing in the human dystrophin gene including use as pharmaceuticals,” in particular “to target exon 53 to induce skipping of the 53<sup>rd</sup> exon.” Ex. 21 (Prosecution History Excerpt of the ’361 patent) at NS00000740. In response, NS did not object to the Examiner’s rejection as unclear or

ambiguous. *See id.* at NS00000753–763. Rather, NS summarized the rejection as the cited prior art encouraging a skilled artisan “to test various sizes of oligomers in an optimization of antisense compounds targeting a known superior *target region*,” specifically “*targeting the 53<sup>rd</sup> exon of the human dystrophin gene* to induce skipping of the 53<sup>rd</sup> exon.” *Id.* at NS00000760 (emphasis added). The claims of the ’361 patent similarly recite “*the 53rd exon in the human dystrophin gene*” with no antecedent basis, confirming a skilled artisan’s ability to understand the “exon 53 of the human dystrophin pre-mRNA” phrase recited in the Wilton patents. Ex. 29 (’361 patent) at claim 1 (emphasis added). Thus, outside the context of this case, NS understood the meaning of these technical terms.

**B. Term 1a: “a base sequence”; Term 1b: “a target region”; and Term 1c: “exon 53 of the human dystrophin pre-mRNA”**

Terms	Sarepta’s Proposed Construction	NS’s Proposed Construction
“a base sequence” (Term 1a)	<i>Not indefinite with respect to Terms 1b and 1c</i>  <i>No construction needed in light of inter alia, the intrinsic evidence and the knowledge of a person of ordinary skill in the art</i>  To the extent construction is needed, Sarepta proposes that each phrase should be given its plain and ordinary meaning, i.e., “a linear sequence of bases” (Term 1a), “the pre-mRNA transcribed from exon 53 of the human dystrophin gene” (Term 1c), and “a segment of the pre-mRNA” (Term 1b)	any sequence of bases that is part of the antisense oligonucleotide
“exon 53 of the human dystrophin pre-mRNA” (Term 1c)		<i>Indefinite</i>
“a target region” (Term 1b)		<i>Indefinite</i>

67. As discussed above, my opinion is that the antisense oligonucleotide phrase as a whole should be considered and construed for proper context. *See supra* ¶¶59–62. But to the extent that these three subparts within the phrase are separately considered, each subpart should

be given its plain and ordinary meaning: (1) “a base sequence” means “a linear sequence of bases”; (2) “exon 53 of the human dystrophin pre-mRNA” means “the pre-mRNA transcribed from exon 53 of the human dystrophin gene”; and (3) “a target region” means “a segment of the pre-mRNA.” *See supra* ¶¶45–62. This is consistent with how these terms are used in the claims, the specification, and the art. *See supra* ¶¶45–62.

68. I understand that NS proposes construing the term “a base sequence” to mean “any sequence of bases that is part of the antisense oligonucleotide.” I disagree with NS’s proposal, which conflicts with other claim limitations. Specifically, using “any” and “part” in its construction, NS implies that only a subset of the bases of the antisense oligonucleotide forms a base sequence, i.e., that there are other bases in the antisense oligonucleotide that do not form a base sequence and are not complementary to the target region of exon 53 of the human dystrophin pre-mRNA. But the claims expressly require the base sequence to be “100% complementary” to the target region. *See supra* ¶¶18–24. A skilled artisan would have understood that all of the bases of the claimed antisense oligonucleotide form a base sequence and are complementary to the target region, not just “any” “part” of the bases as NS’s strained construction implies.

69. NS’s strained reading of the “base sequence” also conflicts with the specification. As discussed above, in Table 1A, the specification defines each exemplary antisense oligonucleotide with its “NUCLEOTIDE SEQUENCE.” *See supra* ¶¶50–51; Ex. 1 (the ’851 patent) at cols. 7–19 (Table 1A). The base sequence of each exemplary antisense oligonucleotide includes *all* of the bases contained within the antisense oligonucleotide, not just some portion of them. *Id.*

70. Lastly, I understand that NS argues that the terms “a target region” and “exon 53 of the human dystrophin pre-mRNA” are indefinite. For the reasons discussed above, I disagree

and believe that both terms are definite since they would be understood by persons skilled in the art. *See supra* ¶¶63–66.

**C. Term 3: “in which uracil bases are thymine bases”**

<b>Sarepta’s Proposed Construction</b>	<b>NS’s Proposed Construction</b>
<p><i>Not indefinite</i></p> <p><i>No construction needed in light of inter alia, the intrinsic evidence and the knowledge of a person of ordinary skill in the art</i></p> <p>To the extent construction is needed, Sarepta proposes that the phrase should be given its plain and ordinary meaning, i.e., “the antisense oligonucleotide has thymine bases instead of uracil bases”</p>	<p><i>Indefinite</i></p>

**1. A Skilled Artisan Would Have Understood that the Claimed “Antisense Oligonucleotide” Has Thymine Bases Instead of Uracil Bases**

71. The clause “in which uracil bases are thymine bases” (referred to here as the thymine bases clause) appears in each claim of the Wilton patents.<sup>2</sup> *See supra* ¶¶18–24. In my opinion, a skilled artisan reading this clause would have understood that it modifies the claimed “antisense oligonucleotide” in its entirety. In other words, the claimed antisense oligonucleotide, not just a portion of it, has thymine bases instead of uracil bases.

**a. Claim Language**

72. Reading the thymine bases clause in the overall context of the claim makes it clear that it modifies the claimed “antisense oligonucleotide” as a whole, not just some unspecified portion of it. Claim 1 of the ’590 patent, which is reproduced below, is illustrative:

---

<sup>2</sup> As such, I have addressed it before Term 2, which only appears in the claims of the ’851 patent.

1. An antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA,

**wherein** the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195),

**in which** uracil bases are thymine bases,

**wherein** the antisense oligonucleotide is a morpholino antisense oligonucleotide, **and**

**wherein** the antisense oligonucleotide induces exon 53 skipping;

or a pharmaceutically acceptable salt thereof.

73. A skilled artisan reading this claim would have understood that it describes four characteristics of the claimed antisense oligonucleotides, each identified by a “wherein” or “in which” clause (highlighted in red and bold above). The first characteristic is the biological role that the claimed antisense oligonucleotide serves, i.e., that *the antisense oligonucleotide* induces exon 53 skipping. The second characteristic is the chemistry of the claimed antisense oligonucleotide, i.e., that *the antisense oligonucleotide* is a morpholino antisense oligonucleotide. The third characteristic is that the base sequence of *the antisense oligonucleotide* has at least 12 consecutive bases derived from SEQ ID NO: 195. The fourth characteristic describes the types of bases in the antisense oligonucleotide, i.e., uracil bases of the *antisense oligonucleotide* are thymine bases. The structure of the claim demonstrates that *the claimed antisense oligonucleotide* contains thymine bases rather than uracil bases.

**b. Exemplary Antisense Oligonucleotides in the Wilton Patents**

74. Exemplary antisense oligonucleotides described in the specification also demonstrate that the claimed antisense oligonucleotides include thymine bases. Table 1A of the Wilton patents discloses exemplary antisense oligonucleotides. It depicts the base sequence of each antisense oligonucleotide as containing uracil bases, not a mixture of uracil bases and thymine bases. Moreover, the table's legend guides a skilled artisan to substitute thymine bases for those uracil bases when making a morpholino, not a combination of uracil bases and thymine bases.

TABLE 1A		
Description of 2'-O-methyl phosphorothioate antisense oligonucleotides that have been used to date to study induced exon skipping during the processing of the dystrophin pre-mRNA. Since these 2'-O-methyl antisense oligonucleotides are more RNA- like, U represents uracil. With other antisense chemistries such as peptide nucleic acids or morpholinos, these U bases may be shown as "T".		
SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5'-3')
1	H8A(-06+18)	GAU AGG UGG <u>UAU</u> CAA CAU <u>CUG</u> <u>UAA</u>
2	H8A (-03+18)	GAU AGG UGG <u>UAU</u> CAA CAU <u>CUG</u>
3	H8A(-07+18)	GAU AGG UGG <u>UAU</u> CAA CAU <u>CUG</u> <u>UAA</u> G
4	H8A(-06+14)	GGU GGU AUC AAC AUC <u>UGU</u> AA
5	H8A(-10+10)	GUA <u>UCA</u> ACA <u>UCU</u> <u>GUA</u> AGC AC

**Figure 8.** Annotated Excerpt of Table 1A of the '851 Patent

75. Reading the claim language in light of this express guidance in the specification, a skilled artisan would have understood the thymine bases phrase to mean that the claimed antisense oligonucleotide, which is a *morpholino* antisense oligonucleotide, has thymine bases instead of uracil bases.

**c. Relevant Prosecution History**

76. My opinions are further supported by the prosecution history of the Wilton patents. *See* Ex. 20 (Prosecution History Excerpt of U.S. Application No. 15/273,772). I understand that U.S. Application No. 15/274,772 (“the ’772 application”) is a direct parent case of the Wilton patents. During prosecution of the ’772 application, the applicant pursued a similarly structured claim directed to an antisense oligonucleotide, setting off the “in which uracil bases are thymine bases” clause from the rest of the claim using a comma (*Id.* at SRPT-VYDS-0091345, SRPT-VYDS-0094178):

1. An antisense oligonucleotide of 25 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA,

**wherein** the base sequence comprises at least 20 consecutive bases of C AUU CAA CUG UUG CCU CCG GUU CU GAAG GUG (SEQ ID NO: 193),

**in which** uracil bases are thymine bases,

**wherein** the antisense oligonucleotide is a morpholino antisense oligonucleotide,

**wherein** the antisense oligonucleotide induces exon 53 skipping, **and**

**wherein** the antisense oligonucleotide is chemically linked to a polyethylene glycol chain;

or a pharmaceutically acceptable salt thereof.

77. In describing the claimed subject matter to the Patent Office, the applicant explained that the claim was directed to “an ***antisense oligonucleotide*** having the following

*elements*: (i) 25 bases comprising a base sequence that is 100% complementary to 25 consecutive bases of exon 53 of the human dystrophin pre-mRNA; (ii) 20 consecutive bases of SEQ ID NO: 193; **(iii) uracil bases are thymine bases**; (iv) the antisense oligonucleotide is a morpholino; (v) the antisense oligonucleotide induces exon 53 skipping; and (vi) the antisense oligonucleotide is chemically linked to a polyethylene glycol chain.” *Id.* at SRPT-VYDS-0094179. In other words, the claimed antisense oligonucleotide had multiple elements, one of which required using thymine bases rather than uracil bases.

78. Similarly, in response the Examiner’s obviousness rejection, the applicant argued that “none of the cited references teach or suggest combining the elements to result in ***the claimed antisense oligonucleotide***,” i.e., “an antisense oligonucleotide of 25 bases, wherein comprises 20 consecutive bases of SEQ ID NO: 193, **and** wherein ***uracil bases are thymine bases***, **and** wherein the antisense oligonucleotide is a morpholino, **and** wherein the resulting antisense oligonucleotide induces exon 53 skipping of the human dystrophin pre-mRNA.” *Id.* at SRPT-VYDS-0094181 (emphasis added except for the underlined words). In other words, the applicant again explained that the claimed antisense oligonucleotide as a whole had thymine bases in place of uracil bases.

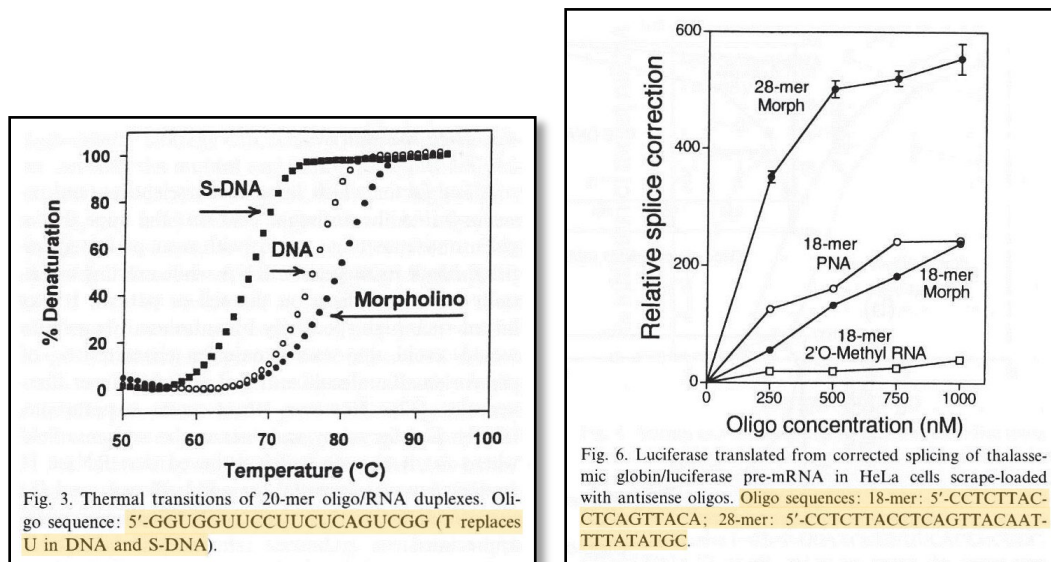
79. These statements from the applicant confirm that the thymine bases clause should be read to modify the claimed “antisense oligonucleotide.”

#### **d. Exemplary Antisense Oligonucleotides in the Prior Art**

80. The use of uracil bases or thymine bases, but not both, is consistent with how antisense oligonucleotides were typically made in the art. Each antisense oligonucleotide is assembled by sequentially adding one nucleotide (or its chemical analog) at a time. Ex. 27 (Summerton 2003) at 217. Using a fixed type of nucleotides simplifies these synthesis processes, avoiding the need to alternate from uracil bases to thymine bases or vice versa in the middle of a synthesis. This also lowers the risk of human error (e.g., incorporating a uracil base as opposed to

a thymine base at a specific location). In the case of morpholinos, the art expressed a preference for thymine bases over uracil bases because of their “positive impact” on the “RNA-binding affinities of the resulting oligos.” *Id.* Knowing these benefits, researchers did not mix uracil bases and thymine bases in a single antisense oligonucleotide, particularly for a morpholino antisense oligonucleotide.

81. A survey of antisense oligonucleotides made and tested in the prior art illustrates this general practice. For example, in an article published in 1999, Dr. James Summerton evaluated several antisense oligonucleotides in both morpholino and non-morpholino formats. Ex. 26 (Summerton 1999) at Abstract. As shown below, Dr. Summerton constructed each antisense oligonucleotide using uracil bases or thymine bases, but not mixtures of both. *Id.* at 147 (Fig. 3), 151 (Fig. 5), 152 (Fig. 6). He further noted that “T replaces U” for certain chemical modifications—similar to how the inventors of the Wilton patents instruct a skilled artisan to construct a morpholino antisense oligonucleotide with thymine nucleotides. *Id.* at 147 (Fig. 3).



**Figure 9.** Annotated Figure 3 and 6 of Ex. 26 (Summerton 1999) at 147, 151

82. **Figure 10** below shows another example. In this article published in 2004, researchers from AVI BioPharma, Inc., the predecessor of Sarepta, published a review article,

summarizing the development of morpholino antisense oligonucleotides as potential therapies. *See* Ex. 6 (Arora 2004) at 431. At least three morpholino antisense oligonucleotides were undergoing clinical trials then, all of which were made with thymine bases exclusively, rather than a mixture of uracil and thymine bases. *Id.* at 435 (Table 3).

ID	Target	Indication	Phase of Development	Sequence 5'-3'
AVI-4126	<i>c-myc</i>	Restenosis	Phase 2 completed	ACGTTG AGG GGC ATC GTC GC
AVI-4126	<i>c-myc</i>	Polycystic Kidney Disease	Phase 1b completed	
AVI-4126	<i>c-myc</i>	Solid Tumors	Phase 1 completed	
AVI-4126	<i>c-myc</i>	Pharmacokinetics	Phase 1 completed	
AVI-4557	CYP3A4	Drug Metabolism	Phase 1 Completed and in progress	CTG GGA TGA GAG CCA TCA CT
AVI-4020	West Nile	West Nile Viral	Phase 1b in progress	CTTAGACATCGAGATCTTCGT G

**Figure 10.** Annotated Table 3 of Ex. 6 (Arora 2004) at 435 (Table 3)

83. In sum, a skilled artisan would have understood that the thymine bases clause refers to the claimed “antisense oligonucleotide” in its entirety. While the meaning of the phrase would have been clear to a skilled artisan, to the extent that it is construed, my opinion is that the phrase should be given its plain and ordinary meaning, i.e., that the antisense oligonucleotide has thymine bases instead of uracil bases.

## 2. The Thymine Bases Clause Is Not Indefinite

84. I understand that NS contends that the thymine bases clause is indefinite because it is purportedly unclear what preceding portion of the claim it modifies. As discussed above, however, a skilled artisan reading the clause in the context of the claims, the specification, and the relevant prosecution history would have understood that the clause refers to the claimed “antisense oligonucleotide” as a whole. *See supra* ¶¶71–83. As such, I disagree that this clause is indefinite.

**D. Term 2: “the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)”**

Sarepta’s Proposed Construction	NS’s Proposed Construction
<p><i>Not indefinite</i></p> <p>Sarepta proposes construing the phrase as: “the target region is within nucleotides +23 to +69 of exon 53 of the human dystrophin pre-mRNA”</p>	<p><i>Indefinite</i></p>

**1. A Skilled Artisan Would Have Understood that the Recited Annealing Sites Identify the Target Region as Within Nucleotides +23 to +69 of Exon 53 of the Human Dystrophin Pre-mRNA**

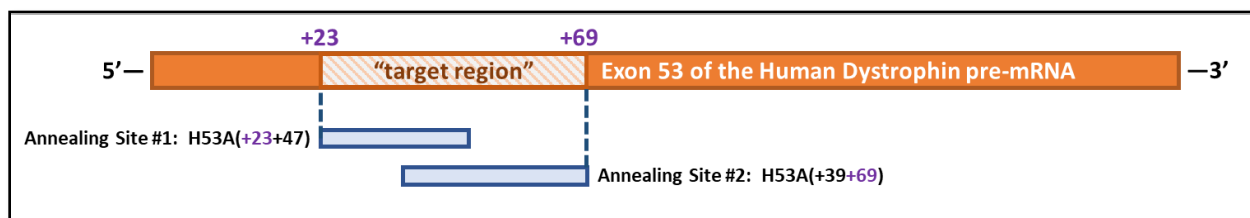
85. The phrase “the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)” (referred to here as the “annealing site” phrase) only appears in the claims of the ’851 patent. *See supra* ¶¶18–24. This phrase should be construed to mean “the target region is within nucleotides +23 to +69 of exon 53 of the human dystrophin pre-mRNA.”

86. A skilled artisan reading the phrase would have understood how to interpret the two specified annealing sites, H53A(+23+47) and H53A(+39+69). For example, the specification explains that in the context of an exon skipping antisense oligonucleotide directed to the human dystrophin pre-mRNA, a target region is expressed using the format “H#A/D(x:y).” Ex. 1 (the ’851 patent) at 22:41–65. The specification explains that “H” designates the species of interest (human in this case), “#” identifies the target dystrophin exon, “A/D” orients the direction of the target region within the exon (either counted from the beginning (the 5’-end) or end (the 3’-end) of the exon), and “(x:y)” represents the annealing coordinates. *Id.* Based on this guidance, a skilled artisan would have understood that annealing site H53A(+23+47) corresponds to nucleotides +23 to +47 (“(+23+47)”) from the beginning (“A”) of exon 53 (“53”) of the human dystrophin pre-mRNA (“H”). Applying the same guidance, annealing site H53A(+39+69) would

have been understood to correspond to nucleotides +39 to +69 (“(+39+69)”) from the beginning (“A”) of exon 53 (“53”) of the human dystrophin pre-mRNA (“H”).

87. Even without this express guidance in the specification, a skilled artisan would have arrived at the same conclusion because this nomenclature system was already known in the art. *See supra* ¶41. Indeed, the specification states that a nomenclature system for antisense oligonucleotides was known in the art, citing a reference published in 2002. Ex. 1 (the ’851 patent) at 22:41–65 (citing Ex. 19 (Mann 2002)). As such, a skilled artisan would have understood that the two recited annealing sites correspond to nucleotides +23 to +47 and nucleotides +39 to +69 of exon 53 of the human dystrophin pre-mRNA, respectively.

88. A skilled artisan would have understood that these overlapping annealing sites define a target region within exon 53 of the human dystrophin pre-mRNA. Specifically, as illustrated below, these two annealing sites identify a region spanning from nucleotide +23 to nucleotide +69 of exon 53 of the human dystrophin pre-mRNA, the beginning marked by the first annealing site H53A(+23+47) and the end marked by the second annealing site H53A(+39+69).



**Figure 11.** “Target Region” of the ’851 Patent

89. This interpretation is supported by the prosecution history of the ’851 patent. During prosecution, the applicant faced an obviousness rejection over prior art references disclosing certain antisense oligonucleotides directed to exon 53 of the human dystrophin pre-mRNA. Ex. 22 (Prosecution History Excerpt of the ’851 patent) at SRPT-VYDS-0004609–11. In response, the applicant explained that the cited prior art did not render the claimed invention

obvious, including “the exon 53 target region +23 to +69.” *Id.* at SRPT-VYDS-0004786. During prosecution of the ’827 patent, the applicant again explained the meaning of the “annealing site” phrase, i.e., as “delineat[ing] a target region . . . spanning from, and including, endpoint H53A+23 to, and including, endpoint H53A+69.” Ex. 23 (Prosecution History Excerpt of the ’827 patent) at SRPT-VYDS-0006276–77. Although the ’827 patent ultimately issued without the “annealing site” language (*see supra* ¶¶23–24), the applicant’s interpretation conforms to how a skilled artisan would have understood it.

90. Notably, NS’s statements made elsewhere further support my opinions. I understand that NS challenged a European patent at the European Patent Office, which stems from the same original patent application that led to the Wilton patents. Claim 1 of that European patent was directed to similar subject matter as the ’851 patent and recited the following (Ex. 10 (European Patent No. 2206781B1) at 43–44):

1. An isolated antisense oligonucleotide that binds to human dystrophin pre-mRNA wherein said oligonucleotide is 20 to 31 nucleotides in length and is an oligonucleotide that is specifically hybridizable to an exon 53 target region of the Dystrophin gene designated as annealing site H53A (+23+47), annealing site H53A (+39+69), or both,  
wherein said antisense oligonucleotide is a morpholino antisense oligonucleotide, and,  
wherein said oligonucleotide induces exon 53 skipping.

91. As shown above, this European patent claim similarly specified that the claimed antisense oligonucleotide was “hybridizable to an exon 53 target region of the Dystrophin gene designated as annealing site H53A(+23+47), annealing site H53A(+39+69), or both.” *Id.* Interpreting the “both” part of this phrase, NS stated that it referred to “the area from nucleotide

+23 until +69”—the same region that a skilled artisan would have identified from reading the “annealing site” phrase of the ’851 patent. Ex. 11 (NS Notice of Opposition) at 4–5.

## **2. The Annealing Site Phrase Is Not Indefinite**


92. I understand that NS contends that this annealing site phrase is indefinite because it is purportedly unclear what portion of exon 53 of the human dystrophin pre-mRNA the claimed target region must be within. As discussed above, a skilled artisan would have understood, with reasonable certainty, that the claimed target region falls within a portion marked by two annealing sites, starting from nucleotide +23 and ending at nucleotide +69 of exon 53 of the human dystrophin pre-mRNA. *See supra* ¶¶86–91. The fact that NS understood a similar phrase as referring to “the area from nucleotide +23 until +69” further supports my opinion. *See supra* ¶¶90–91. As such, I disagree that this phrase is indefinite.

I declare that all statements made herein of my knowledge are true, and that all statements made herein on information and belief are believed to be true. These statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Date:

Jan 4, 2023

By:

  
Cy Aaron Stein, M.D., Ph.D.

# **Appendix A**

**NAME:** Cy Aaron Stein, M.D., Ph.D.

**HOME ADDRESS:** 11 Dolphin Road, New City, NY 10956

**TEL. #:** Cell: (845) 661 3990

E-mail: cyastein@gmail.com

**MARITAL STATUS:** Married - 2 children, three grandchildren

**EDUCATION:**

**College:** Brown University, Providence, Rhode Island, B.A., 1974.

**Graduate:** Stanford University, Stanford, California, Ph.D., Organic Chemistry, 1978.

Thesis: "A Study of Electron Delocalization in Mixed Valence Dithioether Complexes of Rutheniumammines". Thesis Advisor: Dr. Henry Taube (Dr. Taube was the recipient of the Nobel Prize in Chemistry 1983).

**Medical:** Albert Einstein College of Medicine (AECOM) Bronx, New York, M.D. 1982.

**Internship:** The New York Hospital-Cornell Medical Center, 525 E. 68 St., New York, N.Y., 1982-3. Internal Medicine.

**Residency:** The New York Hospital-Cornell Medical Center, 1983-5. Internal Medicine.

**Fellowship:** National Cancer Institute, Bethesda, Maryland, 1985-8.

**Position:** Retired Emeritus Arthur and Rosalie Kaplan Professor and Chair of Medical Oncology and Experimental Therapeutics; Retired Emeritus Professor of Molecular and Cellular Biology, City of Hope Medical Center, Duarte, California, 2020-

**Previous Positions:** Senior Staff Fellow: National Cancer Institute, Bethesda, Maryland, 1988-90.

Assistant Professor of Medicine (7/90-12/95)

Assistant Professor of Medicine and Pharmacology (7/92-12/95):

Associate Professor of Pharmacology and Medicine (1/96-3/03).

Columbia University, College of Physicians and Surgeons

Professor of Medicine and Molecular Pharmacology

Albert Einstein College of Medicine, Bronx, NY 10467

Director, Medical Genitourinary Oncology

Attending Physician

Montefiore Medical Center, Bronx, NY 10467 (3/03-3/12)

Interim Deputy Director, Clinical Research, City of Hope Comprehensive Cancer Center (NCI-designated), 2012-2013

**Diplomate**, American Board of Internal Medicine, (1986)

**Diplomate**, American Board of Oncology (1987)

Maryland Medical License D32665

New York State Medical License 157794

California State Medical License G89157

I maintained a practice of approximately 400 prostate cancer patients at the City of Hope.

#### **AWARDS AND HONORS:**

**Lifetime Achievement Award**, Oligonucleotide Therapeutics Society, 2022. (Award presented in Barcelona, 2023)

Oligonucleotide Therapeutics Society-Hot Papers-June 2016 (paper #130)

Top Cancer Doctors in the US, Newsweek Magazine (2015)

America's Top Doctors for Cancer, Castle Connolly Medical Ltd. (2013, 14, 15)

Faculty of 1000 Biologists Selection (Nov., 2009; paper #116 in bibliography), cited as "exceptional", with F1000 factor 9.0, 5<sup>th</sup> highest of all currently reviewed papers. Ranked #1 in "Hidden Jewels"

Who's Who in Science and Engineering (Marquis, 2016)

Who's Who in America (Marquis, 2010-15)

Who's Who in the World (Marquis, 2011-15, 18-19)

Top Doctors in the LA Area (Oncology), Pasadena Magazine 2013, 14, 15

Top Doctors in Los Angeles (Oncology), Hollywood Reporter 2014, 15

Best Doctors in New York, New York Magazine, 2008-9 (prostate cancer), 2011-12 (prostate and bladder cancer)

New York's "Superdoctors" (top 5%), New York Times Magazine, 2008-9

Best Doctors in the NY Metro Area (2004-11), Castle Connolly Medical Ltd., publisher European Journal of Pharmaceutics and Biopharmaceutics, best paper of 1999 (2000)

American Society for Clinical Investigation (1996)

Irving Assistant Professor of Medicine and Pharmacology, Columbia University (1993-1996)

American Cancer Society, Clinical Career Development Award (1992-1995)

**Co-Editor-in-Chief: Oligonucleotides 1993-2014** (formerly Antisense and Nucleic Acid Drug Development and Antisense Research and Development; Mary-Ann Liebert Publishers)

**Series Editor: Perspectives in Antisense Science** (Kluwer Academic Publishers; four volumes published)

**Deputy Editor:** Molecular Therapy-Nucleic Acids (Cell Press) 2016-2019

**Editorial Advisory Boards:**

*Associate Editor:*

Clinical Cancer Research (American Association for Cancer Research)

*Deputy Editor*

Molecular Therapy-Oncolytics (Cell Press)

*Former Consulting Chief Medical Officer*, Tokai Pharmaceuticals (2009-10, Cambridge, MA).

**Board of Directors/Extended Consultantships:**

ProQR (Leiden, NL); Gentium (Como, IT; Defibrotide); Santaris Pharma (Horsholm, DK; Antisense oligonucleotide technologies); Silence (Berlin, DE; RNAi); Novosom (Halle, DE; Oligonucleotide delivery strategies); ProNai (Kalamazoo, MI; “DNAi”); Genta (Berkeley Heights, NJ; Oblimersen); Dow (Midland, MI; Synthesis and marketplace for oligonucleotides); Altana Pharma (Konstanz, DE; Oligonucleotide silencing technologies); Salus Therapeutics (Salt Lake City, UT; RNAi--acquired by Genta, 2003)

**Oligonucleotide Therapeutics Society** (Founder, 2004, also Treasurer, 2004-2008; ex officio, 2009)

**Professional Organizations:**

American Society for Clinical Oncology

American Society for Gene Therapy (Oligonucleotide Therapeutics Committee, 2005-2008)

**BIBLIOGRAPHY (H Index = 77 calculated by Google)**

1. Stein, C.A. and Morton, T.H. "Methylolithium undergoes halogen exchange faster than it abstracts a carboxylic proton: Stereoselective reduction of 1-methyl-2,2-dibromocyclopropanecarboxylic acid", **Tetrahedron Letters**, 1973, (49) 4933-4936.
2. Stein, C.A., Bouma, S., Carlson, J., Cornelius, C., Maeda, J., Weschler, C., Deutsch, E., and Hodgson, K. "Structural characterization and resulting implications for the mechanism of formation of Bis(2-mercaptoethylamine)ethylenediammine chromium(III) perchlorate,  $[(en)Cr(SCH_2CH_2NH_2)_2]ClO_4$ ", **Inorganic Chemistry**, 1975, (15) 1183-1186.
3. Stein, C.A., Ellis, P.E., Elder, R.C., and Deutsch, E. "Synthesis and characterization of cobalt (III) complexes containing a coordinated selenol. A structural *trans* effect in (2-selenolatoethylamine-*N,Se*) bis(ethylenediammine)cobalt(III) nitrate", **Inorganic Chemistry**, 1976, (15) 1618-1623.
4. Stein, C.A. and Taube, H. "Trimethylsulfonium ion as a ligand: Synthesis and characterization of  $[(NH_3)_5RuS(CH_3)_3](PF_6)_3$ ", **J. Amer. Chem. Soc.**, 1978, (100) 336.
5. Stein, C.A. and Taube, H. "Manifestations of sulfur to sulfur through-space interactions in complex ion spectra", **J. Amer. Chem. Soc.**, 1978, (100), 1635-1637.
6. Stein, C.A. and Taube, H. "Pentaamineruthenium dimethyl chalcogenide complexes", **Inorganic Chemistry**, 1979, (18) 1168-1170.
7. Stein, C.A. and Taube, H. "Ruthenium ammine disulfides", **Inorganic Chemistry**, 1979, (18) 2212-2216.
8. Stein, C.A. and Taube, H. "Manifestations of sulfur to sulfur through-bond interactions in complex ion spectra", **J. Amer. Chem. Soc.**, 1981, (103) 693-695.
9. Stein, C.A., Lewis, N., and Seitz, G. "Long-range intervalence electron tunneling through fully saturated systems", **J. Amer. Chem. Soc.**, 1982, (104) 2596-2599.
10. Stein, C.A., Lewis, N.A., Seitz, G. and Baker, A.D. "Photoelectron spectra of dithiaspirocyclobutane molecules. Electronic spectra of their ruthenium complexes and evidence from CNDO/2 calculations for a hyperconjugating electron-transfer mechanism", **Inorganic Chemistry**, 1983, (22) 1124-1128.
11. Stein, C.A., Ernst, J., Stern, M., Daley, T. and Caspe, W. "Thoracic actinomycosis in a recent tuberculin converter", **Pediatric Infectious Disease**, 1983, (2) 52-55.

12. Baker, A.D., Scharfman, R. and Stein, C.A. "Ultra long-range through-bond interactions in dithiaspirane disulfoxides as revealed by photoelectron spectroscopy", **Tetrahedron Letters**, 1983, (24) 2957-2960.
13. Lalezari, I., and Stein, C.A. "A simple one-step synthesis of 3-amino-2,4(1H,3H)quinazoliniones", **J. Heterocyclic Chem.**, 1984, (21) 5-7.
14. Stein, C.A., Baker, A.D., Lewis, N.A. and White, P.S. "Evidence for through-space interactions between sulfur and nitrogen lone pairs in 2, 2a, 4, 4a, 6, 6a-hexahydro-1,3,5-trithia-7-azacyclopenta[*cd*]-pentalene", **J. Chem. Soc. Dalton Transactions**, 1984, 2073-2075.
15. Stein, C.A., Saville, W., Yarchoan, R., Broder, S. and Gelmann, E., "Suramin and function of the adrenal cortex", **Ann. Intern. Med.**, 1986, (104), 286-287.
16. Feuillan, P., Raffeld, M., Stein, C.A., Lipford, N., Rehnquist, D., Myers, C.E., LaRocca, R.V. and Chrousos, G.P. "Effects of suramin on the function and structure of the adrenal cortex in the cynomolgus monkey", **J. Clin. Endocrinol. Metab.**, 1987, (65) 153-160.
17. Holland, E., Stein, C.A., Palestine, A., La Rocca, R., Chan, C.-C., Kuwabara, T., Myers, C., Thomas, R. McAtee, N. and Nussenblatt, R. "Suramin keratopathy", **Am. J. Ophthalmology**, 1988, (106) 216-221.
18. Stein, C.A., Subasinghe, C., Shinozuka, K. and Cohen, J. "Physicochemical properties of phosphorothioate oligodeoxynucleotides", **Nucl. Acids Res.**, 1988, (16) 3209-3221.
19. Horne, M., Stein, C.A., La Rocca, R. and Myers, C. "Circulating glycosaminoglycan anticoagulants associated with suramin treatment", **Blood**, 1988, (71) 273-279.
20. Stein, C.A., Mori, K., Loke, S.L., Subasinghe, C., Shinozuka, K., Cohen, J.S. and Neckers, L.M. "Phosphorothioate and normal oligodeoxynucleotides with 5'-linked acridine: characterization and preliminary kinetics of cellular uptake", **Gene**, 1988, (72) 333-341.
21. Loke, S., Stein, C.A., Zhang, X., Avigan, M., Cohen, J. and Neckers, L. "Delivery of *c-myc* antisense phosphorothioate oligodeoxynucleotides to hematopoietic cells in culture by liposome fusion: Specific reduction in *c-myc* protein expression correlates with inhibition of cell growth and DNA synthesis", **Curr. Top. Microbiol. Immunobiol.**, 1988, (141) 282-289.
22. Matsukura, M., Zon, G., Shinozuka, K., Stein, C., Mitsuya, H., Cohen, J. and Broder, S. "Synthesis of phosphorothioate analogues of oligodeoxynucleotides and their antiviral activity against human immunodeficiency virus (HIV)", **Gene**, 1988, (72), 343-347.

23. Yano, T., Linehan, M., Anglard, P., Lerman, M., Daniel, L.N., Stein, C.A., Robertson, C.N., LaRoca, R., and Zbar, B. "Genetic changes in human adrenocortical carcinomas", **J. Natl. Cancer Inst.**, 1989, (81) 518-523.
24. Stein, C.A., LaRocca, R., Thomas, R., McAtee, N., and Myers, C.E. "Suramin: An anticancer drug with a unique mechanism of action", **J. Clin. Oncology**, 1989, (7) 499-508.
25. Loke, S.L., Stein, C., Zhang, X., Mori, K., Nakanishi, M., Subasinghe, C., Cohen, J.S., and Neckers, L.M. "Characterization of oligodeoxynucleotide transport into living cells ", **Proc. Natl. Acad. Sci. USA.**, 1989, (86) 3474-3478.
26. Majumdar, C., Stein, C.A., Cohen, J.S., Broder, S. and Wilson, S. "Stepwise mechanism of HIV reverse transcriptase: Primer function of phosphorothioate oligodeoxynucleotide", **Biochemistry**, 1989, (28) 1340-1346.
27. Matsukura, M., Zon, G., Shinozuka, K., Robert-Guroff, M., Shimada, T., Stein, C.A., Mitsuya, H., Wong-Staal, F., Cohen, J. and Broder, S. "Regulation of viral expression of human immunodeficiency virus *in vitro* by an antisense phosphorothioate oligodeoxynucleotide against rev (*art/trs*) in chronically infected cells", **Proc. Natl. Acad. Sci. USA**, 1989, (86) 4244-4249.
28. Gao, W., Stein, C.A., Cohen, J., Dutschman, G. and Cheng, Y.-C. "Effect of phosphorothioate homo-oligodeoxynucleotides on herpes simplex virus type 2-induced DNA polymerase," **Biochemistry**, 1989, (264) 11521-11526.
29. Mori, K., Subasinghe, C., Stein, C.A., and Cohen, J. "Synthesis and properties of novel 5'-linked oligos", **Nucleosides and Nucleotides**, 1989, (8) 649-657.
30. Cazenave, C., Stein, C.A., Loreau, N., Thuong, N.T., Neckers, L., Subasinghe, C., Helene, C., Cohen, J. and Toulme, J.-J. "Comparative inhibition of rabbit globin mRNA translation by modified antisense oligodeoxynucleotides", **Nucl. Acids Res.**, 1989, (17) 4255-4273.
31. Stein, C.A., Matsukura, M., Subasinghe, C., Broder, S., and Cohen, J. "Phosphorothioate oligodeoxynucleotides are potent sequence nonspecific inhibitors of *de novo* infection by HIV", **AIDS Res. and Human Retroviruses**, 1989, (5) 639-646.
32. Mori, K., Boiziau, C., Cazenave, C., Matsukura, M., Subasinghe, C., Cohen, J. Broder, S., Toulme, J.-J., and Stein, C.A. "Phosphoroselenoate oligodeoxynucleotides: Synthesis, physico-chemical characterization, anti-sense inhibitory properties and anti-HIV activity", **Nucl. Acids Res.**, 1989, (17) 8207-8219.
33. Gazdar, A.F., Oie, H., Shackleton, C., Chen, T., Triche, T., Myers, C., Chrousos, G., Brennan, M., Stein, C.A. and LaRocca, R. "Establishment and characterization of a

human adrenocortical carcinoma cell line that expresses multiple pathways of steroid biosynthesis", **Cancer Res.**, 1990, (50) 5488-5496.

34. LaRocca, R., Meer, J., Gilliatt, R., Stein, C.A., Cassidy, J., Myers, C.E., and Dalakas, M., "Suramin-induced polyneuropathy", **Neurology**, 1990 (40) 954-960.

35. Gao, W., Hanes, R., Vazquez-Padua, M., Stein, C.A., Cohen, J., and Cheng, Y.-C. "Inhibition of herpes simplex virus type 2 growth by phosphorothioate oligodeoxynucleotides," **Antimicrobial Agents Chemother.**, 1990 (34), 808-812.

36. LaRocca, R., Stein, C.A., Danesi, R., Jamis-Dow, C.A., Weiss, G.H., and Myers, C. "Suramin in adrenal cancer: Modulation of steroid hormone production, cytotoxicity *in vitro* and clinical antitumor effect." **J. Clin. Endocrinol. Metabolism**, 1990 (71) 497-504.

37. Stein, C.A., Iversen, P.L., Subasinghe, C., Cohen, J., Stec, W.J., and Zon, G. "Preparation of <sup>35</sup>S-labeled polyphosphorothioate oligodeoxynucleotides by use of hydrogen phosphonate chemistry," **Analytical Biochemistry**, 1990 (188) 11-16.

38. Reed, J.C., Stein, C.A., Subasinghe, C., Haldar, S., Croce, C.M., Yum, S., and Cohen, J. "Antisense-mediated inhibition of *BCL2* protooncogene expression and leukemic cell growth and survival: Comparisons of phosphodiester and phosphorothioate oligodeoxynucleotides," **Cancer Res.**, 1990 (50) 6565-6570.

39. Stein, C.A., Neckers, L.M., Nair, B.C., Mumbauer, S., Hoke, G., and Pal, R. "Phosphorothioate oligodeoxynucleotide interferes with binding of HIV-1 gp120 to CD4", **J. Acquired Immune Deficiency Syndromes**, 1991, (4) 686-693.

40. La Rocca, R.V., Stein, C.A., Danesi, R., Cooper, M.R., Uhrich, M., and Myers, C.E. "A pilot study of suramin in the treatment of metastatic renal cell carcinoma," **Cancer**, 1991, (67) 1509-1513.

41. Stein, C.A., Pal, R., DeVico, A.L., Hoke, G., Mumbauer, S., Kinstler, O., Sarngadharan, M.G., and Letsinger, R.L. "Mode of action of 5'-linked cholesteryl phosphorothioate oligodeoxynucleotides in inhibiting syncytia formation and infection by HIV-1 and HIV-2 in vitro", **Biochemistry**, 1991 (30) 2439-2444.

42. Nakajima, M., DeChavigny, A., Johnson, C.E., Hamada, J., Stein, C.A., and Nicolson, G.L. "Suramin: A potent inhibitor of melanoma heparanase and invasion", **J. Biol. Chem.**, 1991 (266) 9661-9666.

43. Chou, T.-C., Zhu, Q.-Y., and Stein, C.A. "Differential alteration of the anti-HIV-1 effect of phosphorothioate oligodeoxynucleotide S-dC28 by AZT, interferon-alpha, and dextran sulfate", **AIDS Research Human Retrovir.**, 1991, (7) 943-951.

44. Pal, R., Mumbauer, S., Hoke, G., LaRocca, R., Myers, C., Sarngadharan, M.G., and Stein, C.A. "Effect of Evans Blue and Trypan Blue on Syncytia Formation and Infectivity of Human Immunodeficiency Virus Type I and Type II In Vitro", **AIDS Research Human Retrovir.**, 1991, (7) 537-543.
45. Myers, C., Cooper, M., Stein, C.A, LaRocca, R., Walther, M.M., Weiss, G., Choyke, P., Dawson, N., Steinberg, S., Uhrich, M.M., Cassidy, J., Kohler, D.R., Trepel, J., and Linehan, W.M. "Suramin: a novel growth factor antagonist with activity in hormone-refractory metastatic prostate cancer", **J. Clin. Oncology**, 1992, (10) 881-889. (See accompanying editorial "Suramin awakes?").
46. Marshall, W.S., Beaton, G., Stein, C.A., Matsukura, M., and Caruthers, M.H. "Inhibition of human immunodeficiency virus activity by phosphorodithioate oligodeoxycytidine", **Proc. Natl. Acad. Sci.**, 1992, (89) 6265-6269.
47. Wade, T., Kasid, A., Stein, C.A., LaRocca, R.V., Sargent, E.R., Gomella, L., Myers, C., and Linehan, W. M. "Suramin interference with transforming growth factor- $\beta$  inhibition of human renal cell carcinoma in culture", **J. Surg. Res.**, 1992 (53)195-198.
48. LaRocca, R.V., Cooper, M.R., Stein, C.A., Kohler, D., Uhrich, M., Weinberger, E. and Myers, C.E. "A pilot study of suramin in the treatment of progressive refractory follicular lymphomas". **Ann. Oncology**, 1992, (3), 571-573.
49. Saxon, M., Schieren, I., Zhang, L.-M., Tonkinson, J.L., and Stein, C.A. "Stimulation of calcium influx in HL60 cells by cholesteryl-modified homopolymer oligodeoxynucleotides", **Antisense Res. Devel.**, 1992, (2) 243-250.
50. Yakubov, L.A., Vlassov, V.V., Zhang, L.-M. and Stein, C.A. "Interaction of oligonucleotides with a recombinant CD4 Receptor," **Doklady Akademii Nauk Rossiya**, 1992 (327) 593-597
51. Krieg, A.M., Tonkinson, J., Matson, S., Zhao, Q., Saxon, M., Zhang, L.-M., Bhanja, U., Yakubov, L. and Stein, C.A. "Modification of antisense phosphodiester oligodeoxynucleotides by a 5' cholesteryl moiety increases cellular association and improves efficacy," **Proc. Natl. Acad. Sci. USA**, 1993, (90), 1048-1052.
52. Stein, C.A., Tonkinson, J.L., Zhang, L.-M., Yakubov, L. Gervasoni, J., Taub, R., and Rotenberg, S.A. "Dynamics of the internalization of phosphodiester oligodeoxynucleotides in HL60 cells," **Biochemistry**, 1993, (32), 4855-4861.
53. Stein, C.A., Cleary, A.M., Yakubov, L., and Lederman, S. "Phosphorothioate oligodeoxynucleotides bind to the third variable loop domain (v3) of human immunodeficiency virus Type 1 gp120", **Antisense Res. Devel.**, 1993, (3) 19-31.

54. Yakubov, L., Khaled, Z., Zhang, L.-M., Truneh, A., Vlassov, V., and Stein, C.A. "Oligodeoxynucleotides interact with recombinant CD4 at multiple sites". **J. Biol. Chem.**, 1993, (268), 18818-18823.
55. Perez, J.R., Li, Y., Stein, C.A., Majumder, S., van Oorschot, A., and Narayanan, R. "Sequence-independent induction of Sp1 transcription factor activity by phosphorothioate oligodeoxynucleotides", **Proc. Natl. Acad. Sci. USA**, 1994, (91) 5957-5961.
56. Archambault, D., Stein, C.A., and Cohen, J.S. "Phosphorothioate oligonucleotides inhibit the replication of lentiviruses and type D retroviruses, but not that of type C retroviruses", **Archives Virol.**, 1994, (139) 97-109.
57. Tonkinson, J.L., and Stein, C.A. "Patterns of intracellular compartmentalization, trafficking and acidification of 5'-fluorescein labeled phosphodiester and phosphorothioate oligodeoxynucleotides in HL60 cells", **Nucl. Acids Res.**, 1994, (22) 4268-4275.
58. Tonkinson, J.L., Guvakova, M., Khaled, Z., Lee, J., Yakubov, L., Marshall, W.S., Caruthers, M.H., and Stein, C.A. "Cellular pharmacology and protein binding of phosphoromonothioate and phosphorodithioate oligodeoxynucleotides: A comparative study", **Antisense Res. Devel.**, 1994, (4) 269-278.
59. LaRocca, R., Cooper, M., Danesi, R., Stein, C.A., and Myers, C.E. "Suramin therapy for malignant thymoma: A case report", **European J. Cancer** 1994, (30) 718-719.
60. Khaled, Z., Rideout, D., O'Driscoll, K.R., Petrylak, D., Cacace, A., Patel, R., Chiang, L., Rotenberg, S., and Stein, C. A. "Effects of suramin-related and other clinically therapeutic polyanions on protein kinase C activity", **Clin. Cancer Res.**, 1995, (1) 113-122.
61. Stein, C.A., Khan, T., Khaled, Z., and Tonkinson, J. "Cell surface binding and cellular internalization properties of suramin, a novel antineoplastic agent", **Clin. Cancer Res.**, 1995, (1) 509-517.
62. Guvakova, M.A., Yakubov, L.A., Vlodavsky, I., Tonkinson, J.L., and Stein, C.A. "Phosphorothioate oligodeoxynucleotides bind to basic fibroblast growth factor, inhibit its binding to cell surface receptors, and remove it from low affinity binding sites on extracellular matrix", **J. Biol. Chem.**, 1995, (270) 2620-2627.
63. Su, Z.-Z., Yemul, S., Stein, C.A., and Fischer, P. "c-fos is a positive regulator of carcinogen enhancement of adenovirus transformation", **Oncogene**, 1995, (10) 2037-2049.
64. Henderson, G. B., and Stein, C.A. "5'-Cholesteryl phosphorothioate oligodeoxynucleotides: Potent inhibition of methotrexate transport and antagonism of

methotrexate toxicity in cells containing the reduced-folate carrier", **Nucl. Acids Res.**, 1995, (23) 3726-3731.

65. Bishop, W., Lin, J., Wen, J., Stein, C.A., and Krieg, A. "Interruption of a TGF- $\alpha$ -EGF receptor autocrine loop in Caco-2 cells by TGF- $\alpha$  antisense oligodeoxynucleotides", **Gastroenterology**, 1995, (109) 1882-1889.

66. Sharma, H., Sokoloski, J., Sartorelli, A., Stein, C.A., Nichols, G., Khaled, Z., Ratan, R., Perez, J., Maltese, J., Telang, N., and Narayanan, R. "Differentiation of immortal cells inhibits telomerase activity: Implications in cancer", **Proc. Natl. Acad. Sci. USA**, 1995, (92) 12343-12346.

67. Benimetskaya, L., Tonkinson, J., Koziolkiewicz, M., Karwowski, B., Guga, P., Zeltser, R., Stec, W., and Stein, C.A. "Binding of phosphorothioate oligodeoxynucleotides to basic fibroblast growth factor, recombinant soluble CD4, laminin and fibronectin is P-chirality independent", **Nucl. Acids Res.**, 1995, (23) 4239-4245.

68. Khaled, Z., Benimetskaya, L., Khan, T., Zeltzer, R., Sharma, H., Narayanan, R., and Stein, C.A. "Multiple mechanisms may contribute to the cellular antiadhesive effects of phosphorothioate oligodeoxynucleotides", **Nucl. Acids Res.**, 1996, (24) 737-745.

69. Wang, W., Chen, H., Schwartz, A., Stein, C.A. and Rabbani, L. "Sequence-independent inhibition of *in vitro* vascular smooth muscle proliferation, migration, and *in vivo* neointimal formation by phosphorothioate oligodeoxynucleotides", **J. Clin. Invest.**, 1996, (98) 443-450.

70. Lederman, S., Sullivan, G., Benimetskaya, L., Lowy, I., Land, K., Khaled, Z., Cleary, A., Yakubov, L., and Stein, C.A. "Polydeoxyguanine motifs in a 12-mer phosphorothioate oligodeoxynucleotide augment binding to the v3 loop of HIV-1 gp120 and potency of HIV-1 inhibition independently of G-tetrad formation", **Antisense Nucleic Acid Drug Devel.** (formerly Antisense Research and Development), 1996, (6) 281-289.

71. Wang, W., Chen, H., Warshofsky, M., Schwartz, A., Stein, C.A., and Rabbani, L. "Phosphorothioate oligodeoxynucleotide sequence-independent inhibition of vascular smooth muscle cell adhesion and fibrinolysis", **Antisense Nucleic Acid Drug Devel.** 1997, (7) 101-108.

72. Benimetskaya, L., Loike, J., Khaled, Z., Loike, G., Silverstein, S., Cao, L., El-Khoury, J., Kai, T.-Q., and Stein, C.A. "Mac-1 (CD11b/CD18) is a cell surface oligodeoxynucleotide binding protein", **Nature-Medicine**, 1997, (3) 414-420.

73. Rockwell, P., O'Connor, W., King, K., Goldstein, N., Zhang, L.M. and Stein, C.A. "Cell surface perturbations of the EGF and VEGF receptors by phosphorothioate oligodeoxynucleotides", **Proc. Natl. Acad. Sci. USA**, 1997, (94) 6523-6528.

74. Liu, C.-Y., and Stein C.A. "Taxol and estramustine-induced modulation of human prostate cancer cell apoptosis via alteration in bcl-xL and bak expression", **Clin. Cancer Res.**, 1997, (3) 2039-2046.
75. Benimetskaya, L., Berton, M., Kolbanovsky, A., Benimetsky, S. and Stein, C.A. "Formation of a G-tetrad and higher order structures correlates with biological activity of the RelA (p65) "antisense" oligodeoxynucleotide", **Nucl. Acids Res.**, 1997, (25) 2648-2656.
76. Wang, W., Chen, H., Schwartz, A., Cannon, P., Stein, C.A., and Rabbani, L. "Chain-length dependency, time course, and distribution of the non-sequence-specific effects of phosphorothioate oligodeoxynucleotides on in vivo neointimal formation", **Antisense Nucl. Acid Drug Devel.**, 1997, (7) 559-566.
77. Liu, C.-Y., Rubin, M., Omene, C., Lederman, S., and Stein, C.A. "Prostate cancer cells constitutively secrete Fas Ligand in vitro", **Clin. Cancer Res.** 1998, (4) 1803-1811.
78. Wang, W., Chen, H., Sun, J., Benimetskaya, L., Schwartz, A., Cannon, P., Stein, C.A., and Rabbani, L. "A comparison of guanosine-quartet inhibitory effects versus cytidine homopolymer inhibitory effects on rat neointimal formation", **Antisense Nucl. Acid Drug Devel.** 1998, (8) 227-236.
79. Yakubov, L., Kit, Y., Richter, V., Andreeva, A., Karamyshev, V., Stein, C.A., and Vlassov, V. "The extracellular domain of the CD4 receptor is a protein kinase", **FEBS Lett.**, 1998 (431) 45-48.
80. Benimetskaya, L., Takle, G., Vilenchik, M., Lebedeva, I., Miller, P., and Stein, C.A. "Cellular delivery of antisense oligodeoxynucleotides by a novel cationic porphyrin vehicle", **Nucl. Acids Res.**, 1998, (26) 5310-5317.
81. Berton, M., Benimetskaya, L., Weil, R., Zwillenberg, L., Stein, C.A., and Gurny, R. "Uptake of oligonucleotide loaded nanoparticles in prostatic cancer cells and their intracellular localization", **Euro. J. Pharmaceutics Biopharmaceutics**, 1999, (47) 119-123.
82. Berton, M., Alleman, E., Stein, C.A., and Gurny, R. "Highly loaded nanoparticulate carrier using an hydrophobized antisense oligonucleotide complex", **Euro. J. Pharmaceutical Sciences**, 1999, (9)163-170
83. Khaled, Z., Ho, Y., Benimetskaya, L., Deckelbaum, R. and Stein, C.A. "ω-6 Polyunsaturated fatty acid stimulated cellular internalization of phosphorothioate oligodeoxynucleotides: Evidence for protein kinase C-ζ dependency", **Biochem. Pharmacol.** 1999, (58) 411-423.

84. Yamamoto, H., Soh, J.-W., Klein, M., Zhang, L.-M., Schieren, I., Stein, C.A., and Weinstein, I.B. "Antisense to Rb inhibits growth and induces apoptosis in a colon carcinoma line", **Clin. Cancer Res.**, 1999, (5) 1805-1815.
85. Simon, A., Giedd, K., Schwartz, A., Wang, W., Sun, J., Marboe, C., Stein, C.A., and Rabbani, L. "Porous balloon delivery of SdC28 does not prevent restenosis in the porcine coronary artery model of balloon injury", **Antisense Nucleic Acid Drug Devel.**, 1999, (9) 549-553.
86. Miao, H., Elkin, M., Aingorn, E., Ishai-Michaeli, R., Stein, C.A., and Vlodavsky, I. "Inhibition of heparanase activity and tumor metastasis by laminarin sulfate and synthetic phosphorothioate oligodeoxynucleotides", **Int. J. Cancer** 1999, (83) 424-31
87. Ma, M., Benimetskaya, L., Lebedeva, I., Dignam, J., Takle, G., and Stein, C.A. "Intracellular mRNA cleavage induced via activation of RNase P by nuclease-resistant external guide sequences (EGS)", **Nature-Biotechnol.**, 2000, (18) 58-61.
88. Lebedeva, I., Rando, R., Ojwang, J., Cossum, P., and Stein, C.A. "Bcl-xL in prostate cancer cells: Effects of overexpression and downregulation on chemosensitivity" **Cancer Res.**, 2000, (60) 6052-6060.
89. Vilenchik, M., Benimetsky, L., Miller, P., and Stein, C.A. "Evidence for higher order structure formation by the c-myb 18-mer phosphorothioate antisense (codons 2-7) oligodeoxynucleotide: Potential relationship to antisense c-myb inhibition", **Antisense Nucleic Acid Drug Devel.**, 2001, (11) 87-97.
90. Koziolkiewicz, M., Gendaszewska, M., Maszewska, M., Stein, C.A. and Stec, W. J. "The mononucleotide-dependent, non-antisense mechanism of action of phosphodiester and phosphorothioate oligonucleotides depends on the activity of an ecto-5'-nucleotidase", **Blood**, 2001, (98) 995-1002.
91. Su, Z.-z., Lebedeva, I., Stein, C.A., Reed, J.C., Dent, P., and Fisher, P.B. A combinatorial approach for selectively inducing programmed cell death in human pancreatic cells", **Proc. Natl. Acad. Sci USA**, 2001, (98) 10332-10337
92. Lebedeva, I., Rando, R., Ojwang, J., Cossum, P., and Stein, C.A. "Chemosensitization of bladder carcinoma cells by bcl-xL antisense oligonucleotides", **J. Urology**, 2001 (166) 461-469.
93. Berton, M., Turelli, P., Trono, D., Stein, C.A., and Gurny, R. "Inhibition of HIV-1 in cell culture by oligonucleotide-loaded nanoparticles", **Pharmaceutical Res.**, 2001, (18) 1096-1101.
94. Benimetskaya, L., Miller, P., Benimetsky, S., Stec, W., Guga P., Beaucage, S., Halperin, A., and Stein, C.A. "Inhibition of potentially anti-apoptotic proteins by antisense PKC- $\alpha$  (Isis 3521) and antisense Bcl-2 (G3139) phosphorothioate

oligodeoxynucleotides: Relationship to the decreased viability of T24 bladder and PC3 prostate cancer cells”, **Mol. Pharmacol.**, 2001, (60) 1296-1307.

95. Benimetskaya, L., Guzzo-Pernell, N., Ting, S., Lai, J., Miller, P., and Stein, C.A. “Protamine-fragment peptides linked to an SV40 nuclear localization signal deliver oligonucleotides that produce antisense effects in prostate and bladder carcinoma cells”, **Bioconjugate Chem.**, 2002, (13) 177-187.

96. Vilenchik, M., Raffo, A., Benimetskaya, L., Shames, D., and Stein, C.A. “Antisense RNA downregulation of bcl-xL expression in prostate cancer cells leads to diminished rates of cellular proliferation and resistance to cytotoxic chemotherapeutic agents”, **Cancer Res.**, 2002, (62) 2175-2183.

97. Roberts, K.E., Hamale-Bena, D., Stein, C.A., and Cole, R. “Pulmonary Tumor Embolism: A case and review of the literature”, **Amer. J. Med.**, 2003, (115) 228-232.

98. Lai, J., Benimetskaya, L., Santella, R., Wang, Q., Miller, P. and Stein, C.A. “G3139 (Oblimersen) may inhibit prostate cancer cell growth in a partially bis-CpG-dependent , non-antisense manner”, **Molecular Cancer Therap.**, 2003, (2) 1031-1043

99. Lebedeva, I., Sarkar, D., Su, Z., Kitada, S., Dent, P., Stein, C.A., Reed, J., and Fisher, P. “Bcl-2 and bcl-xL differentially protect human prostate cancer cells from induction of apoptosis by melanoma differentiation associated gene-7, mda-7/IL24”, **Oncogene**, 2003, (22) 8758-8773.

100. Raffo, A., Lai, J., Miller, P., Stein, C.A., and Benimetskaya, L. “Antisense RNA downregulation of Bcl-2 expression in DU145 prostate cancer cells does not diminish the cytostatic effects of G3139 (Oblimersen)”, **Clin. Cancer Res.**, 2004, (10) 3195-3206.

101. Benimetskaya, L., Wittenberger, T., Stein, C.A., Hofmann, H.-P., Weller, C., Lai, J., Miller, P., and Gekeler, V. “Changes in gene expression induced by G3139 in prostate carcinoma cells are recapitulated at least in part by treatment with interferon- $\beta$  and - $\gamma$ ”, **Clin. Cancer Res.**, 2004, (10) 3678-3688.

102. Benimetskaya, L., Lai, J., Wu, S., Hua, E., Khvorova, A., Miller, P. and Stein, C.A. “Relative Bcl-2 independence of drug induced cytotoxicity and resistance in 518A2 melanoma cells”, **Clin. Cancer Res.**, 2004, (10) 8371-8379.

103. Lai, J., Benimetskaya, L., Khvorova, A., Wu, S., Hua, E., Miller, P., and Stein, C.A. “Phosphorothioate oligodeoxynucleotides and G3139 induce apoptosis in 518A2 melanoma cells”, **Molecular Cancer Therap.** 2005, (4) 305-315.

104. Benimetskaya, L., Miller, P., and Stein, C.A. “518A2 melanoma cells are protected by G3139 and other anti-neoplastic agents against the cytotoxic effects of DTIC”, **Oligonucleotides**, 2005, (15) 206-214.

105. Anderson, E., Miller, P., Ilsley, D., Marshall, W., Khvorova, A., Stein, C.A., and Benimetskaya, L. "Gene profiling study of G3139 and Bcl-2-targeting siRNAs identifies a unique G3139 molecular signature", **Cancer Gene Therapy**, 2006, (13) 406-414.
106. Gekeler, V., Gimmnich, P., Hofmann, H.-P., Grebe, C., Romele, M., Leja, A., Baudler, M., Benimetskaya, L., Gonser, B., Pieses, W., Maier, T., Wagner, T., Sanders, K., Beck, J., Hanauer, G., and Stein, C.A., "G3139 and other CpG-containing immunostimulatory phosphorothioate oligodeoxynucleotides are potent suppressors of the growth of human tumor xenografts in nude mice," **Oligonucleotides**, 2006, (16) 83-93.
107. Lai, J., Tan, W., Benimetskaya, L., Miller, P., Colombini, M., and Stein, C.A. "A pharmacologic target of G3139 in melanoma cells may be the mitochondrial VDAC", **Proc. Natl. Acad. Sci. USA**, 2006, (103) 7494-7499.
108. Benimetskaya, L., Ayyanar, K., Kornblum, N., Castanotto, D., Rossi, J., Wu, S., Lai, J., Popova, N., Brown, B., Miller, P., McMicken, H., Chen, Y., and Stein, C.A. "Bcl-2 protein in 518A2 melanoma cells *in vivo* and *in vitro*", **Clin. Cancer Res.**, 2006, (12) 4940-4948.
109. Tan, W., Lai, J., Miller, P., Stein, C.A., and Colombini, M. "G3139 reduces mitochondrial outer membrane permeability to ADP", **Amer. J. Physiol. Cell Physiol.**, 2007, (292) C1388-C1397.
110. Lai, J., Brown, B., Voskresenskiy, A., Vonhoff, S., Tan W., Colombini, M., Weeratna, R., Miller, P., Benimetskaya, L., and Stein, C.A. "Comparison of D-G3139 and its enantiomer L-G3139 in melanoma cells demonstrates minimal *in vitro* but dramatic *in vivo* chiral dependency", **Molecular Therapy**, 2007, (15) 270-278.
111. Zhao, H., Peng, P., Yu, D., Longley, C., Zhang, Y., Borowski, V., Mehlig, M., Reddy, P. Xia, J., Borchard, G., Lipman, J., Benimetskaya, L., and Stein, C.A. "Delivery of G3139 using releasable PEG-linkers: Impact on pharmacokinetic profile and anti-tumor efficacy", **J. Controlled Release**, 2007, (119) 143-152.
112. Stein, C.A., Goel, S. and Ghavamian, R. "Hepatitis and rhabdomyolysis in a patient with hormone refractory prostate cancer treated with ketoconazole on concurrent lovastatin therapy", **Invest. New Drugs**, 2007, (25) 277-278.
113. Tan, W., Loke, Y.-H., Stein, C.A., Miller, P. and Colombini, M. "Phosphorothioate oligonucleotides block the VDAC channel", **Biophys. J.**, 2007 (93) 1184-1191.
114. Benimetskaya, L., Wu, S., Voskresenskiy, A., Echart, C., Zhou, J.-F., Shin, J., Iacobelli, M., Richardson, P., Ayyanar, K., and Stein, C.A. "Angiogenesis-alteration by Defibrotide: Implications for its mechanism of action in severe hepatic veno-occlusive disease", **Blood**, 2008, (112) 4343-4352.  
See comment: Lazarus, H. and McCrae, K. "SOS! Defibrotide to the rescue" **Blood**, 2008, (112) 3924-3925.

115. Stein, C.A., Wu, S., Voskresenskiy, A., Zhou, J.-F., Shin, J., Miller, P., and Benimetskaya, L. "G3139, an anti-Bcl-2 antisense oligomer that binds heparin-binding growth factors and collagen I, alters in-vitro endothelial cell growth and tubular morphogenesis", **Clinical Cancer Res.**, 2009, (15) 2797-2807.
116. Stein, C.A., Hansen, B., Lai, J., Soifer, H., Wu, S., Voskresenskiy, A., Hoeg, A., Worm, J., Hedtjarn, M., Soulemanian, N., Miller, P., Castanotto, D., Benimetskaya, L., Oerum, H., and Koch, T. "Efficient gene silencing by delivery of locked nucleic acid antisense oligonucleotides, unassisted by transfection reagents", **Nucleic Acids Res.** 2009 <http://doi:10.1093/nar/gkp841>.
117. Agarwala, S., Keilholz, U., Gilles, E., Bedikian, A., Wu, J., Kay, R., Stein, C.A., Itri, L., Suci, S., and Eggermont, A. "LDH correlation with survival in advanced melanoma from two large, randomized trials: Oblimersen (GM301) and EORTC18951", **Eur. J. Cancer**, 2009, (45) 1807-1814.
118. Michaelson, M.D., Bellmunt, J., Hudes, G., Goel, S., Lee, R., Kantoff, P., Stein, C.A., Lardelli, P., Pardos, I., Kahatt, C., Nieto, A., Cullell-Young, M., Lewis, N., Smith, M. Multicenter phase II trial of trabectin in patients with metastatic castration resistant prostate cancer, **Annals of Oncology**, 2011, doi:10.1093/annonc/mdr399
119. Soifer, H., Souleimanian, N., Wang, S., Voskresenskiy, A., Collak, F., Cinar, B., and Stein, C.A. "Direct regulation of androgen receptor activity in prostate cancer cells by potent CYP17 inhibitors", **J. Biol. Chem.**, 2012, (287), 3777-3787.
120. Souleimanian, N., Deleavy, G., Soifer, H., Wang, S., Damha, M., Tiemann, K., and Stein, C.A. "Antisense 2'-fluoro, 2'-deoxyarabino nucleic acids (2'F-ANA) oligonucleotides: In vitro gymnotic silencers of gene expression whose potency is enhanced by fatty acids", **Molecular Therapy-Nucleic Acids**, 2012, 1, e43; doi:10.1038/mtna.2012.35.
121. Pal, S., Twardowski, P., and Stein, C.A. "Long-term treatment of an octogenarian with castrate resistant prostate cancer (CRPC) with cabazitaxel", **Clinical Genitourinary Cancer**, 2012, (10), 274-276 2012
122. Pal, S., Yamzon, J., Sun, V., Carmichael, C., Saikia, J., Ferrell, B., Frankel, P., Yamzon, J., Twardowski, P., Stein, C.A., Margolin, K. "Paclitaxel-based high-dose chemotherapy with autologous stem cell rescue for relapsed germ cell tumor: Clinical outcome and quality of life in long-term survivors," **Clinical Genitourinary Cancer**, 2013, (11) 121-127.
123. Bhagat, T., Zhou, L., Sokol, L., Gundabolu, K., Gordon, S., Mantzaris, I., Gligich, O., Yu, Y., Nischal, S., Bhattacharyya, S., Polineni, J., Chrysafakis, G., Schinke, C., Bhatia, K., Wickrema, A., Pellagatti, A., Boultonwood, J., Kambhampati, S., Parekh, S., Steidl, U., Stein, C., Ju, W., Liu, G., List, A., Bitzer, M., and Verma, A. miR-21

mediates hematopoietic suppression in MDS by activating TGF- $\beta$  signaling, **Blood**, 2013 (121), 2875-2881.

124. Williams, S., Atalla, C., Ghavamian, R., Stein, C.A., Hoenig, D. “‘Extreme’ renal preservation: Neoadjuvant chemotherapy and percutaneous resection for upper tract urothelial carcinoma in a patient with solitary kidney—a case report,” **J Endourology**, 2013 (27), 427-431.

125. James L. Mohler, Philip W. Kantoff, Andrew J. Armstrong; Robert R. Bahnson, Michael Cohen, Anthony Victor D’Amico, James A. Eastham, Charles A. Enke, Thomas A. Farrington; Celestia S. Higano, Eric Mark Horwitz, Mark H. Kawachi, Michael Kuettel, Richard J. Lee, Gary R. MacVicar, Arnold W. Malcolm, David Miller, Elizabeth R. Plimack, Julio M. Pow-Sang, Sylvia Richey, Mack Roach III, Eric Rohren, Stan Rosenfeld, Eric J. Small, Sandy Srinivas, Cy Stein, Seth A. Strobe, Jonathan Tward, Patrick C. Walsh, Dorothy A. Shead, and Maria Ho. “Prostate Cancer, Version 1.2014 Featured Updates to the NCCN Guidelines” **J. Natl. Comprehensive Cancer Network** 2013 (11), 1471-1479.

126. Mohler, J.L., Kantoff, P.W., Armstrong, A.J., Bahnson, R.R., Cohen, M., D’Amico, A.V., Eastham, J.A., Enke, C.A., Farrington, T.A., Higano, C.S., Horwitz, E.M., Kane, C.J., Kawachi, M.H., Kuettel, M., Kuzel, T.M., Lee, R.J., Malcolm, A.W., Miller, D., Plimack, E.R., Pow-Sang, J.M., Raben, D., Richey, S., Roach, M., 3rd, Rohren, E., Rosenfeld, S., Schaeffer, E., Small E.J., Sonpavde, G., Srinivas, S., Stein, C., Strobe, S.A., Tward, J., Shead, D.A., Ho, M. National Comprehensive Cancer Network. “Prostate Cancer, Version 2.2014 Featured Updates to the NCCN Guidelines”. **J. Natl. Comprehensive Cancer Network** 2014 (12) 686-718.

127. Rodriguez, R., Woolliscroft, M., Abdul-Shukkur, E., Forgey, R., McGovren, P., Endert, G., Wagner, A., Holewa, D., Aboukameel, A., Gill, R., Bisgaier, C., Messmann, R., Izbicka, E., Streeper, R. Wick, M., Stiegler, G., Stein, C.A., Monsma, D., Webb, C., Sooch, M., Panzner, S., Mohammed, R., Goodwin, N., Al-Katib, A. “Development and antitumor activity of a Bcl-2 targeted single-stranded DNA oligonucleotide”, **Cancer Chemotherapy and Pharmacology**, 2014 (74), 151-166.

128. Pal, S., Gartrell, B., Olsson, C., Stein, C.A. “Metastatic adrenocortical carcinoma with a prolonged response to mitotane,” **Oncology**, July, 2014, 624-625, 642.

129. He, M., Tew, B., Stein, C., Rideout, D., Pal, S., and Jones, J.O. “A synthetic chemical approach to improve the understanding of the multiple activities of abiraterone,” **Clinical and Experimental Pharmacology** 5:173. <http://doi10.4172/2161-1459.1000173>, 2015.

130. Castanotto, D., Lin, M., Kowolik, C., Wang, L., Ren, X.-Q., Soifer, H.S., Koch, T., Hansen, B.R., Armstrong, B., Wang, Z., Bauer, P., Rossi, J., and Stein, C.A. “A distinct

cytoplasmic pathway for antisense-mediated gene silencing in mammalian cells,” **Nucleic Acids Research**, 2015, (43) 9350-9361.

131. Castanotto, D., Lin, M., Kowolik, C., Koch, T., Hansen, B.R., Oerum, H., and Stein, C.A. “Protein kinase C- $\alpha$  is a critical protein for antisense oligonucleotide mediated silencing in mammalian cells,” **Molecular Therapy**, 2016, (24) 1117-25.

132. Zhang, X.-W., Castanotto, D., Horne, D., Nam, S., and Stein, C.A. “6BIO enhances oligonucleotide activity in cells: A potential combinatorial anti-androgen receptor therapy in prostate cancer cells,” **Molecular Therapy**, 2017, (25) 79-91.

133. Bohr, H., Shim, I., Stein, C.A., Oerum, H., Hansen, H., and Koch, T. “Electronic structures of LNA phosphorothioate oligonucleotides”, **Molecular Therapy-Nucleic Acids**, 2017, (8) 428-441

134. Papangelou, M., Olszanski, A., Stein, C., Zhang, Y., Bosch, B., and Nemeth, P. “The effect of food on the absorbtion of abiraterone acetate from a fine particle dosage form: A randomized crossover trial in healthy volunteers,” **Oncology and Therapy**, 2017, doi:10.1007/s40487-017-0054-2.

135. Hussaini, A., Olszanski, A., Stein, C., Bosch, B., and Nemeth, P. “Impact of an alternative steroid of the relative bioavailability and bioequivalence of a novel formulation of abiraterone acetate versus the originator,” **Cancer Chemotherapy and Pharmacology**, 2017 doi: 10.1007/s00280-017-3360-3

136. Stein, C.A., Levin, R., Given, R., Higano, C., Nemeth, P., Bosch, B., Chapas-Reed, J., and Dreicer, R. “Randomized Phase 2 therapeutic equivalence study of abiraterone acetate fine particle formulation evaluating serum testosterone and prostate-specific antigen levels in patients with metastatic castration-resistant prostate cancer: The STAAR study.” **Urologic Oncology**, 2017 (36) 81e9-81.e16

137. Zhang, X., Castanotto, D., Shemi, A., and Stein, C.A. “Ammonium and arsenite (As III) are potent, inexpensive facilitators of oligonucleotide function when delivered by gymnosia,” **Nucleic Acids Res.**, 2018 (46) 3612-3624.

138. Castanotto, D., Zhang, X., Alluin, J., Zhang, Y., Rüger, J., Armstrong, B., Rossi, J., Riggs, A., and Stein, C.A. “A Stress-Induced Response Complex (SIRC) shuttles oligonucleotides and miRNAs to the nucleus.” **Proc. Natl. Acad. Sci. USA**, 2018, (115) 756-765

139. Dorff, T. B., Stein, C., Kortylewski, M., Posadas, E., Synold, T., Quinn, D. “Evaluating changes in immune function and bone microenvironment during radium-223 treatment of patients with castration-resistant prostate cancer.” **Cancer Biotherapy and Radiopharmaceuticals**. (7) 485-489, 2020.

140. Chapas-Reed, J., Stein, C., Squittieri, N., and Concepcion, R. "One-year, open label extension study on the safety and efficacy of abiraterone acetate fine particle formulation in patients with metastatic castration-resistant prostate cancer." **Clinical Medicine Insights: Urology**, (13), 1-7, 2020.

141. Castanotto, D., Zhang, X., Ruger, A., Alluin, J., Sharma, R., Pirrotte, P., Joenson, L., Ioannou, S., Nelson, M., Vikesa, J., Hansen, B., Koch, T., Jensen, M., Rossi, J., and Stein, C.A. "A multifunctional LNA oligonucleotide-based strategy blocks AR expression and transactivation activity in PCa cells." **Molecular Therapy—Nucleic Acids**, (2021).

### BOOK CHAPTERS, REVIEWS AND EDITORIALS

1. Mertelsmann, R., Welte, K., Sykora, K.-W., Kolitz, J., Halloway, K., Fiedler, W., Miller, G., Merluzzi, V., Venuta, S., Engert, A., Stein, C., Polivka, A. and Levi, E. "Treatment of Cancer with Interleukin 2", in **Development, Evaluation and Applications of Biologically Active Substances in Cancer Therapy**, P. Schuff-Werner and K. Pfitzenmaier, eds., W. Zuckschwerdt Verlag, Munich, 1985, p. 137-157.

2. Stein, C.A., and Cohen, J., "Oligodeoxynucleotides as inhibitors of gene expression: A review", **Cancer Research**, 1988, (48), 2659-2668.

3. Stein, C.A. and Cohen, J. "Antisense Compounds: Potential role in cancer therapy", in DeVita, V., et al., eds. **"Important Advances in Oncology-1989"** J.B. Lippincott, New York, 1989, p. 79-97.

4. Stein, C.A., and Cohen, J.S. "Phosphorothioate oligodeoxynucleotide analogues", in **"Oligodeoxynucleotides: Anti-sense Inhibitors of Gene Expression,"** J. Cohen, ed. McMillen Press, London, 1989, p 97-118.

5. Stein, C.A., LaRocca, R.V., and Myers, C.E. "Suramin: An old compound with new biology" in **Principles and Practice of Oncology Updates**, Number 5, V. Devita, et al., eds. J.B. Lippincott, New York, 1990, p. 419-431.

6. LaRocca, R.V., Stein, C.A., and Myers, C.E. "Suramin-prototype of a new generation of antitumor compounds." **Cancer Cells**, 1990, (2), 106-115.

7. LaRocca, R., Stein, C.A., Danesi, R., and Myers, C. "Suramin, a novel antitumor compound", **J. Steroid Biochem. Molecular Biol.**, 1990, (37), 893-899.

8. Myers, C.E., LaRocca, R.V., Cooper, M.R., Danesi, R., Jamis-Dow, C.A., Stein, C.A., and Linehan, W.M. "Role of suramin in cancer biology and treatment", in **Molecular Foundations of Oncology**, S. Broder, ed., J. Wiley, Baltimore, 1991, pp 419-431.

9. Stein, C.A., Tonkinson, J.L., and Yakubov, L. "Phosphorothioate oligodeoxynucleotides -- Anti-sense inhibitors of gene expression?" **Pharmacology and Therapeutics**, 1991, (52), 365-384.
10. Stein, C.A. "Antisense oligodeoxynucleotides -- Promises and pitfalls", **Leukemia**, 1992, (6), 967-974.
11. Stein, C.A. and Pal, R. "Anti-HIV compounds with membrane-oriented specificity - early results" in **Cellular Membrane: A Key to Disease Processes**, C. Onishi and S. Onishi, eds., CRC Press, Boca Raton, FL, 1992, Chapter 14, pp. 337-353.
12. Stein, C.A. "Antisense inhibition of gene expression", in **Principles and Practice of Oncology**, 4th Ed., V. DeVita, et al., eds., J.B. Lippincott, New York, 1993, 2646-2655.
13. Stein, C.A. "Suramin: A novel antineoplastic agent with multiple potential mechanisms of action", **Cancer Research**, 1993, (52), 2239-2248.
14. Stein, C.A. and Cheng, Y.-C. "Antisense oligonucleotides: Is the bullet really magical?", **Science**, 1993, (261) 1004-1012.
15. Tonkinson, J., and Stein, C.A. "Antisense nucleic acids -- prospects for antiviral intervention", **Antiviral Chemistry and Chemotherapy**, 1993, (4), 193-200.
16. Tonkinson, J., and Stein, C.A. "The cellular internalization of antisense oligodeoxynucleotides", **Antiviral News**, 1993, (1), 39-40.
17. Rideout, D., and Stein, C.A. "Suramin and its analogs as antineoplastic agents", **Drugs of the Future**, 1994, (19), 161-174.
18. Stein, C.A. and Narayanan, R. "Antisense oligonucleotides", **Current Opinion in Oncology**, 1994, (6), 587-594.
19. Stein, C.A. and Krieg, A. Editorial: "Problems in interpretation of data derived from *in vitro* and *in vivo* use of antisense oligodeoxynucleotides" **Antisense Research and Development**, 1994, (4), 67-69.
20. Stein, C.A. "Antisense oligodeoxynucleotides" in **Biologic Therapy of Cancer, 2nd ed.**, V. DeVita, et al., eds. J.B. Lippincott, New York, 1995, 759-773.
21. Stein, C.A. "Does antisense exist?" Commentary in **Nature-Medicine**, 1995, (1), 1119-1121. See letter to the editor and response in **Nature-Medicine**, 1996, (2), 131-132.
22. Tonkinson, J., and Stein, C.A. "Antisense oligonucleotides-The potential for clinical application", **Cancer Investigation**, 1996, (14), 54-65.

23. Stein, C.A. and Narayanan, R. "Cellular Biochemistry of Oligodeoxynucleotides", in **Perspectives in Drug Discovery and Design**, P. Anderson et al., eds., 1996, (4), 1-10.
24. Gewirtz, A., Stein, C.A. and Glazer, P. "Facilitating Oligonucleotide Delivery: Helping Antisense Deliver On Its Promise", Commentary, **Proc. Natl. Acad. Sci. USA**, 1996, (93), 3161-3163.
25. Stein, C.A. "Phosphorothioate Oligodeoxynucleotides-A Question of Specificity" **Trends in Biotechnology**, 1996, (14), 147-149.
26. Stein, C.A. and Cheng, Y.-C. "Antisense Inhibition of Gene Expression", in **Principles and Practice of Oncology**, 5th Ed., V. DeVita, et al., eds., J.B. Lippincott, New York, 1997, 3059-3074
27. Stein, C.A. "Antisense c-myc Phosphorothioate Oligodeoxynucleotides-A Question of Mechanism", *invited editorial*, **J. Natl. Cancer Inst.**, 1996, (88), 391-393.
28. Stein, C.A. "Exploiting the Potential of Antisense: Beyond Phosphorothioate Oligodeoxynucleotides", **Chemistry and Biology**, 1996, (3), 319-323.
29. Stein, C.A. "Controversies in the Cellular Pharmacology of Oligodeoxynucleotides," **Antisense and Nucl. Acid Drug Devel.**, 1997, (7), 207-210
30. Stein, C.A. and Krieg, A. "Non-sequence specific effects of phosphorothioate oligodeoxynucleotides. In **Antisense Technology: A Practical Approach**, C. Lichtenstein and W. Nellen, eds. Oxford University Press, Oxford, UK, 1997, 241-264.
31. Stein, C.A. "Mechanisms of Intracellular Oligodeoxynucleotide Internalization", **Ciba Foundation Symposium #209**, London, UK, 1997, 79-93.
32. Stein, C.A. and Krieg, A., Eds. **"Applied Antisense Oligonucleotide Technology"**, J. Wiley and Sons, Inc., New York, NY, 1998, 540 pp.
33. Hanss, B., Stein, C.A. and Klotman, P. "Cellular Internalization of Oligodeoxynucleotides", in Stein, C.A. and Krieg, A., eds. **"Applied Antisense Oligonucleotide Technology"**, J. Wiley and Sons, Inc., New York, NY, 1998, 111-128.
34. Stein, C.A. "How to design an antisense oligodeoxynucleotide experiment: A consensus approach", **Antisense and Nucleic Acid Drug Development**, 1998, (8), 129-132.
35. Lebedeva, I. and Stein, C.A. "Phosphorothioate Oligodeoxynucleotides", in Rabbani, L., ed. **"Applications of Antisense Therapies to Restenosis"**, Volume I of "Antisense Oligodeoxynucleotides", Stein, C.A., series ed. Kluwer Academic Publishers, Norwell, MA, 1999, 99-118.

36. Wyatt, J. and Stein, C.A. "G-quartet Inhibitory Effects of Phosphorothioate Oligodeoxynucleotides", in Rabbani, L., ed. "**Applications of Antisense Therapies to Restenosis**", Volume I of "Antisense Oligodeoxynucleotides", Stein, C.A., series ed. Kluwer Academic Publishers, Norwell, MA, 1999, 133-140.
37. Rabbani, L., and Stein, C.A. "Potential use of oligonucleotides in restenosis," **Antisense and Nucleic Acid Drug Development**, 1999, (8), 487-492.
38. Stein, C.A. "In Vitro Delivery of Oligonucleotides and Some Antisense Experimental Do's and Dont's," **Biochem. Biophys. Acta**, 1999, (1489) 45-52.
39. Takle, G., and Stein, C.A. "Cellular Uptake of Oligodeoxynucleotides" in **Pharmaceutical Aspects of Oligonucleotides**, 1999, P. Couvreur and C. Malvy, eds. Taylor & Francis, London, UK, p. 201-212.
40. Benimetskaya, L., Tonkinson, J., and Stein, C.A. "Determination of Cellular Internalization of Oligonucleotides, in **Methods in Enzymology: Antisense Techniques**, 1999, M. Ian Phillips, ed., Academic Press, Orlando, p. 287-297.
41. Stein, C.A. "Keeping the Biotechnology of Antisense in Context", Commentary in **Nature-Biotechnology**, 1999, (17), 209.
42. Stein, C.A. "Hybridization Prediction Gets to First Base", **Nature-Biotechnology**, News and Views, 1999 (17), 751-752.
43. Stein, C.A. "Molecular and Cellular Mechanisms of Action of Taxanes", **Seminars in Oncology**, 1999, (26) 15suppl. 3-7.
44. Stein, C.A. "Antisense Oligodeoxynucleotides" in **Biologic Therapy of Cancer, 3rd ed.**, S. Rosenberg, ed. J.B. Lippincott, New York, 2000, 812-824.
45. Lebedeva, I., and Stein, C.A. "Antisense in Cancer: Recent Advances", **BioDrugs**, 2000, (13), 195-216.
46. Stein, C.A. "Is Irrelevant Cleavage the Price of Antisense Efficacy?", **Pharmacol. Therap.**, 2000 (85), 231-236.
47. Lebedeva, I., Benimetskaya, L., Stein, C.A., and Vilenchik, M. "Delivery of Oligonucleotides to Cells", **European J. of Pharmaceutics and Biopharmaceutics**, 2000, (50), 101-119
48. Stein, C.A. *Invited Book Review*: "Better Than Prozac? Antisense Technology in the Central Nervous System", **Trends in Pharmacol. Sci.**, 2000, (21), 230-231

49. Lebedeva, I. and Stein, C.A. "Antisense Oligonucleotides: Promise and Reality", **Annual Review of Pharmacology and Toxicology**, 2001 (41), 403-419
50. Stein, C.A. and Lebedeva, I. "Antisense to Bcl-2 in Therapy for Cancer", in **Gene Therapy of Cancer: Translational Approaches from Preclinical Studies to Clinical Implementation**, Gerson, S., and Lattime, E., eds. 2001, 313-330
51. Stein, C.A. and Lebedeva, I. "Antisense Downregulation of the Apoptosis-Related Bcl-2 and Bcl-xL Proteins: A New Approach to Cancer Therapy", **J. Molecular Biol. (Russ.)**, 2001 (34), 875-887.
52. Stein, C.A. "Guidelines for the Experimental Use of Antisense Oligonucleotides," **J. Clin. Invest.**, 2001 (108) 641-644.
53. Stein, C.A. "Antisense that Comes Naturally", News and Views in **Nature Biotechnology**, 2001 (19), 737
54. Koehn, R., Rossi, J., and Stein, C.A. "In Defense of Antisense", Letter to the Editor in **Science**, 2001 (293), 1047-1048.
55. Dias, N., and Stein, C.A. "The Use of Antisense Oligonucleotides to Validate Gene Function", **Molecular Cancer Therapeutics**, 2001 (1), 347-355.
56. Dias, N., and Stein, C.A. "Update on Antisense Oligonucleotide Therapeutics-bcl-2 and PKC- $\alpha$ ", **Eur. J. Pharmaceuticals and Biopharmaceutics**, 2002 (54), 263-269.
57. Benimetskaya L., and Stein, C.A. "Antisense Therapy: New Advances" **Clinical Prostate Cancer**, 2002, (1) 20-30.
58. Stein, C.A. "Antisense Technology Becomes Therapeutics" **Science and Medicine**, 2003, (8) 318-327.
59. Liu, W., Bulgaru, A., Haigentz, M., Stein, C.A., Perez-Soler, R., and Mani, S. The bcl-2-family of protein ligands as cancer drugs: The next generation of therapeutics. **Curr. Med. Chem.-Anti-Cancer Agents**, 2003 (3), 217-223.
60. Nichols, G. and Stein, C.A. "Modulation of the activity of bcl-2 in Waldenstrom's Macroglobulinemia using antisense oligonucleotides" **Semin. Oncol.** 2003, (30) 297-299.
61. Wang, L., Prakash, R., Stein, C.A., Koehn, R., and Ruffner, D. "Progress in the Delivery of Therapeutic Oligonucleotides: Organ/cellular Distribution and Targeted Delivery of Oligonucleotides *In Vivo*", **Antisense and Nucleic Acid Drug Development**, 2003, (13) 169-189
62. Cho-Chung, Y., Gewirtz, A., and Stein, C.A. (eds). Therapeutic Oligonucleotides. **Ann. NY Acad Sci.**, 2003 (1002).

63. Stein, C.A., Dias, N., Benimetskaya, L., Jepsen, J., Lai, J., and Raffo, A. "LY900003 (Isis 3521) and G3139 (Genasense; Oblimersen)-Phosphorothioate Antisense Oligonucleotides with Pleiotropic Mechanisms of Action." **Nucleic Acid Therapeutics in Cancer**, A. Gewirtz, ed., 2004, 171-191.
64. Stein, C.A., Mani, S., Benimetskaya, L. "Combinations of Chemotherapy with Antisense Approaches Against bcl-2", in **Combination Cancer Therapy: Modulators and Potentiators**, G. Schwartz, ed., Humana Press, 2005, p. 207-228
65. Stein, C.A., Benimetskaya, L., and Mani, S. "Antisense Inhibition of Gene Expression", in **Principles and Practice of Oncology**, 7th Ed., V. DeVita, et al., eds., J.B. Lippincott, New York, 2005, p. 2891-2898.
66. Stein, C.A. and Rossi, J. "A Critical Assessment of the Potential of Short Interfering RNA Therapeutics", **Drug Discovery Today: Technologies**, 2005, [www.drugdiscoverytoday.com](http://www.drugdiscoverytoday.com); <http://www.sciencedirect.com> (2) 27-31.
67. Cho-Chung, Y., Gewirtz, A., and Stein, C.A. (eds). Therapeutic Oligonucleotides—Transcriptional and Translational Strategies for Silencing Gene Expression. **Ann. NY Acad Sci.**, 2005 (1058)
68. Benimetskaya, L., Lai, J., Khvorova, A., Wu, S., Miller, P. and Stein, C.A. "Induction of Apoptosis by G3139 in Melanoma Cells", **Ann. NY Acad. Sci.**, 2005 (1058) 235-245.
69. Stein, C.A., Benimetskaya, L., and Mani, S. "Antisense Strategies for Oncogene Inactivation", **Semin. Oncol.** 2005, (32) 563-572.
70. Kornblum, N., Ayyanar, K., Benimetskaya, L., Richardson, P., Iacobelli, M., and Stein, C.A. "Defibrotide, a Polydisperse Mixture of Single-Stranded Phosphodiester Oligonucleotides with Life-Saving Activity in Severe Hepatic Venous Occlusive Disease: Clinical Outcomes and Potential Mechanisms of Action", **Oligonucleotides**, 2006 (16), 105-114.
71. Stein, C.A. "The Genasense 'Learning Curve'", Letter to the Editor, **Nature Biotechnology**, 2007, (25) 972-973.
72. Stein, C.A. "Genasense: It Ain't Over Till the Fat Lady Sings", Editorial, **Oligonucleotides**, 2007 (17) 345-348.
73. Stein, C.A. Kornblum, N., Lai, J., and Benimetskaya, L. "Genasense (G3139): An Antisense Bcl-2 Oligodeoxyribonucleotide with Substantial Clinical Activity and a Complex Mechanism of Action". In **Therapeutic Oligonucleotides**, J. Kurreck, ed., Biomolecular Science, Royal Society of Chemistry, 2008, p. 23-42

74. Stein, C.A., Benimetskaya, L., Kornblum, N., and Mani, S. "Antisense Agents", in **Principles and Practice of Oncology**, 8th Ed., V. DeVita, et al., eds., J.B. Lippincott, New York, 2008.
75. Stein, C.A., and Colombini, M. "Specific VDAC Inhibitors: Phosphorothioate Oligonucleotides," **J. Bioenerg. Biomemb.**, 2008, (40), 157-162.
76. Stein, C.A., Hoehn, B., and Rossi, J. "Oligonucleotide Therapeutics". In **Principles of Anticancer Drug Development**, E. Garrett-Mayer, et al., eds. Springer Press, 2011, p. 565-587.
77. Stein, C.A., and Soifer, H. "Antisense Inhibition of Gene Expression." In **Principles and Practice of Oncology**, 9th Ed., V. DeVita, et al., eds., J.B. Lippincott, New York, 2011.
78. Soifer, H., Koch, T., Hansen, B., Hoeg, A., Oerum, H., and Stein, C.A. "Silencing of Gene Expression by Gymnotic Delivery of Antisense Oligonucleotides" in M. Kaufmann and C. Klinger, eds., **Functional Genomics: Methods and Protocols: Methods in Molecular Biology**, Springer Press, 2011, (815), 333-346.
79. Stein, C.A. and Goel, S. "Therapeutic Oligonucleotides—The Road Not Taken," invited commentary in **Clinical Cancer Research**, 2011, (17), 6369-6372.
80. Twardowski, P. and Stein, C.A. "Beyond the Abstract-Direct Regulation of Androgen Receptor Activity in Prostate Cancer Cells by Potent CYP17 Inhibitors." **UroToday.com**, July 2, 2012
81. Hu, J., Hsu, J., Bergerot, P., Yuh, B., Stein, C.A., Pal, S. "Preoperative Therapy for Localized Prostate Cancer: A Comprehensive Overview." **Maturitas**, 2013, (74), 3-9.
82. Pal, S., Stein, C.A., Sartor, O. "Enzalutamide for the Treatment of Prostate cancer," **Expert Opinion in Pharmacotherapy**, 2013, (14), 679-685.
83. Eroglu, Z., Stein, C.A., Pal, S. "Targeting Angiopoietin-2 Signaling in Cancer Therapy, **Expert Opinion in Investigational Drugs**, 2013, (22), 813-825.
84. Stein, C.A. "Delivery of Antisense Oligonucleotides to Cells: A Consideration of Some of the Barriers," **Chemistry Today**, 2014, (32), 4-7.
85. Stein, C.A., Castanotto, D., Kowolik, C., and Lin, M. "Antisense Oligonucleotide Therapy in Cancer," **Expert Opinion in Medical Oncology**, 2014, (26), 584-589
86. Kurreck, J. and Stein, C.A. "**Molecular Medicine**," J. Wiley and Sons, Berlin, DE, 2016, 385 p. (Note: This is a two-author book); [www.amazon.com](http://www.amazon.com).

87. Stein, C.A. “Treatment Sequencing in Metastatic Castrate-Resistant Prostate Cancer (mCRPC): A Clinical Commentary, **Clinical Genitourinary Cancer**, 2015, (13), 407-409.
88. Stein, C.A., Castanotto, D., Krishnan, A., and Nikolaenko, L. “Defibrotide (Defitelio<sup>TM</sup>): A New Addition to the Stockpile of FDA-Approved Oligonucleotide Drugs.” **Molecular Therapy-Nucleic Acids**, 2016, 5, e346; doi:10.1038/mtna.2016.42.
89. Stein, C.A. Review of Khleif, S., Rixe, O., Skeel, R. “Skeel’s Handbook of Cancer Therapy, 9<sup>th</sup> Edition,” Wolters Kluwer, Philadelphia, 2016. **Doody’s Review Service** (on-line) Available: <http://www.doody.com> (Accessed August 1, 2016).
90. Stein, C.A. “Eteplirsen is Approved for Duchenne’s Muscular Dystrophy: **Molecular Therapy Nucleic Acids**, 2016, (24), 1884-1885.
91. Stein, C.A. and Castanotto, D. “FDA-Approved Oligonucleotide Therapies-2017,” **Molecular Therapy—Special Issue Commemorating the 20<sup>th</sup> Anniversary of the ASGT**, 2017, (25) 1069-1075.
92. Stein, C.A. “Learning from Suramin: A case study of NCI’s much-hyped cancer drug that crashed and burned—35 years ago. **The Cancer Letter**, 2019 (45) 4-10, August 9.
93. Stein, C.A., Rueger, J., Ioannou, S., and Castanotto, D. “Oligonucleotides to the (gene) rescue: FDA Approvals 2017-2019,” **Trends in Pharm. Sci. (TIPS)**, in press, 2020.
94. Dorff, T., Lyou, Y., and Stein, C.A. Invited Commentary: “Defining Value in Metastatic Prostate Cancer – What is the Cost of Living Longer and Better?” **J. Oncol. Prac.**, in press, 2020.
95. Stein, C.A. Life is Loss (And how I tolerated it when I became a house officer: A personal memoir In: Loss and Grief, p. 196-208. Ed. by M. Loscalzo and M. Forstein, Oxford University Press, New York, 2023

**PATENTS ISSUED:**

1-3) Inhibitors for replication of retroviruses and for the expression of oncogene products. **U.S. patent nos. 5,264,423; 5,276,019; and 5,286,717.** Issued November 23, 1993; January 4, 1994; and February 15, 1994. These three patents were licensed in 2002 to Aventis/Genta for \$10,000,000 and formed part of the IP surrounding G3139 (Genasense).

4) Use of suramin to treat rheumatologic diseases. **U.S. patent no. 5,158,940.** Issued, October 27, 1992.

5) A method of treating glaucoma. **U. S. patent no. 5,545,626.** Issued, August 13, 1996

6) Phosphorothioate oligonucleotides that bind to the v3-loop and uses thereof. **U.S. patent no. 5,756,710.** Issued May 26, 1998.

7) SdC28 as an anti-restenosis agent after balloon injury. **U.S. patent no. 5,854,223.** Issued December 29, 1998.

8) Methods of affecting intracellular phosphorylation of tyrosine using phosphorothioate oligonucleotides and anti-angiogenic and anti-proliferative uses thereof. **U.S. patent no.- 6,030,955.** Issued August 27, 1999

9) Antisense heparanase oligonucleotides. **U.S. patent no. 6,770,753** Issued October, 2004

10) Novel peptide constructs for the delivery of antisense oligonucleotides, **U.S. patent no. 6,867,043** Issued, March 15, 2005.

11) Oligonucleotide inhibitors of Bcl-xL, **U.S. patent no. 7,074,769** Issued, July 11, 2006

12) Treatment of prostate cancer, **U.S. patent no. 8,791,094** Issued, July 29, 2014

13) TKI permeability enhancers. Filed: Nov. 17, 2014

14) Splice-switching agents and strategy for preventing expression of activity of the androgen receptor and other unwanted genes. Filed: Sept. 14, 2016

15) Methods for intracellular delivery and enhancement of gene targeting. Filed: Nov. 15, 2016

### **Historical Fiction Novels**

Cy Stein, **Rocket's Red Glare**, Abeel St. Press (New City, NY) 2020;

Cy Stein, **The Medicus Codex**, Insomnia Press (Londonderry, NH), 2020;  
[www.amazon.com](http://www.amazon.com);

Cy Stein, **Becoming Caligula**, Abeel St. Press (New City, NY) 2022

Cy Stein, **A Time for Lies—Roy Cohn and the Poisoning of America**, Abeel St. Press  
(New City, NY) 2022

# **Appendix B**

**Materials Considered**

<b>Exhibit Number</b>	<b>Description</b>
1	U.S. Patent No. 9,994,851
2	U.S. Patent No. 10,227,590
3	U.S. Patent No. 10,266,827
4	Aartsma-Rus et al., “Targeted Exon Skipping as a Potential Gene Correction Therapy for Duchenne Muscular Dystrophy,” <i>Neuromuscul. Disord.</i> (2002) 12: S71-S77 (“Aartsma-Rus 2002”)
5	Aartsma-Rus et al., “Antisense-Mediated Exon Slipping: A Versatile Tool with Therapeutic and Research Applications,” <i>RNA</i> (2007) 13(10): 1609-1624 (“Aartsma-Rus 2007”)
6	Arora et al., “Neutrally Charged Phosphorodiamidate Morpholino Antisense Oligomers: Uptake, Efficacy and Pharmacokinetics,” <i>Curr. Pharm. Biotechnol.</i> (2004) 5(5): 431-439 (“Arora 2004”)
7	Alberts et al., <i>Molecular Biology of the Cell</i> 191-234, 299-374 (4th ed. 2002) (“Alberts 2002”)
8	Botvin Madorsky at al., “Psychosocial Aspects of Death and Dying in Duchenne Muscular Dystrophy,” <i>Arch. Phys. Med. Rehabil.</i> (1984) 65(2): 79-82 (“Botvin Madorsky 1984”)
9	Exondys 51 <sup>®</sup> (Eteplirsen) Prescribing Information (Revised: 1/2022) (“Eteplirsen Label”)
10	European Patent No. 2206781 B1
11	Nippon Shinyaku Co. Ltd., Opposition Against European Patent No. 2206781 B1 (“NS Notice of Opposition”)
12	FDA News Release dated September 19, 2016 (“FDA 2016”)
13	FDA News Release dated December 12, 2019 (“FDA 2019”)
14	FDA News Release dated August 12, 2020 (“FDA 2020”)
15	Vyondys 53 <sup>®</sup> (Golodirsen) Prescribing Information (Revised: 2/2021) (“Golodirsen Label”)
16	Hoffman et al., “Conservation of the Duchenne Muscular Dystrophy Gene in Mice and Humans,” <i>Science</i> (1987) 238(4825): 347-350 (“Hoffman 1987”)
17	Interference No. 106,007, Decision dated May 12, 2016
18	Koenig et al., “The Complete Sequence of Dystrophin Predicts a Rod-Shaped Cytoskeletal Protein,” <i>Cell</i> (1988) 53(2):219-228 (“Koenig 1988”)
19	Mann et al., “Improved Antisense Oligonucleotide Induced Exon Skipping in the Mdx Mouse Model of Muscular Dystrophy,” <i>J. Gene Med.</i> (2002) 4(6): 644-654
20	Prosecution History Excerpt of U.S. Application No. 15/273,772
21	Prosecution History Excerpt of U.S. Patent No. 9,708,361
22	Prosecution History Excerpt of U.S. Patent No. 9,994,851
23	Prosecution History Excerpt of U.S. Patent No. 10,266,827
24	Summerton et al., “Morpholino Antisense Oligomers: Design, Preparation, and Properties,” <i>Antisense Nucleic Acid Drug Dev.</i> (1997) 7(3): 187-195 (“Summerton 1997(a)”)

Exhibit Number	Description
25	Summerton et al., “Morpholino and Phosphorothioate Antisense Oligomers Compared in Cell-Free and In-Cell Systems,” <i>Antisense Nucleic Acid Drug Dev.</i> (1997) 7(2): 63-70 (“Summerton 1997(b)”)
26	Summerton, “Morpholino Antisense Oligomers: The Case for an RNase H-Independent Structural Type,” <i>Biochim. Biophys. Acta</i> (1999) 1489(1): 141-158 (“Summerton 1999”)
27	Summerton, “Mopholinos and PNAs Compared,” <i>Lett. Pept. Sci.</i> (2003) 10: 215-236 (“Summerton 2003”)
28	Suzuki et al., “Gene Therapy for Duchenne Muscular Dystrophy,” <i>Future Neurol.</i> (2006) 2(1): 87-96 (“Suzuki 2007”)
29	U.S. Patent No. 9,708,361
30	van Deutekom et al., “Antisense-Induced Exon Skipping Restores Dystrophin Expression in DMD Patient Derived Muscle Cells.” <i>Hum. Mol. Genet.</i> (2001) 10(15): 1547-1554 (“van Deutekom 2001”)
31	Viltepso® (Viltolarsen) Prescribing Information (Revised: 3/2021) (“Viltolarsen Label”)
32	Wilton et al., “Specific Removal of the Nonsense Mutation from the Mdx Dystrophin mRNA Using Antisense Oligonucleotides,” <i>Neuromuscul. Disord.</i> (1999) 9(5):330-338 (“Wilton 1999”)
33	Wilton et al., “Antisense Oligonucleotides, Exon Skipping and the Dystrophin Gene Transcript,” <i>Acta Myol.</i> (2005) 24(3): 222-229 (“Wilton 2005”)
34	Aartsma-Rus et al., “Less is More: Therapeutic Exon Skipping for Duchenne Muscular Dystrophy,” <i>Lancet Neurol.</i> (2009) 8(10): 873-875 (“Aartsma-Rus 2009”)
35	Swanson, “FDA Approves Sarepta Therapeutics’ Vyondys 53 for Treatment of DMD Amenable to Exon 53 Skipping,” Muscular Dystrophy Association: Quest (December 13, 2019), <a href="https://mdaquest.org/fda-approves-sarepta-therapeutics-vyondys-53-for-treatment-of-dmd-amenable-to-exon-53-skipping/">https://mdaquest.org/fda-approves-sarepta-therapeutics-vyondys-53-for-treatment-of-dmd-amenable-to-exon-53-skipping/</a> (“MDA 2019”)

**CERTIFICATE OF SERVICE**

I hereby certify that on January 5, 2023, copies of the foregoing were caused to be served upon the following in the manner indicated:

Amy M. Dudash, Esquire  
MORGAN, LEWIS & BOCKIUS LLP  
1201 North Market Street, Suite 2201  
Wilmington, DE 19801  
*Attorneys for Plaintiff*

*VIA ELECTRONIC MAIL*

Amanda S. Williamson, Esquire  
Christopher J. Betti, Esquire  
Krista Vink Venegas, Esquire  
Maria E. Doukas, Esquire  
Michael T. Sikora, Esquire  
Zachary Miller, Esquire  
Guylaine Haché, Ph.D.  
MORGAN, LEWIS & BOCKIUS LLP  
110 North Wacker Drive, Suite 2800  
Chicago, IL 60606  
*Attorneys for Plaintiff*

*VIA ELECTRONIC MAIL*

Eric Kraeutler, Esquire  
MORGAN, LEWIS & BOCKIUS LLP  
1701 Market Street  
Philadelphia, PA 19103  
*Attorneys for Plaintiff*

*VIA ELECTRONIC MAIL*

Jitsuro Morishita, Esquire  
MORGAN, LEWIS & BOCKIUS LLP  
16F, Marunouchi Building,  
2-4-1 Marunouchi, Chiyoda-ku  
Tokyo, 100-6316 Japan  
*Attorneys for Plaintiff*

*VIA ELECTRONIC MAIL*

*/s/ Megan E. Dellinger*

---

Megan E. Dellinger (#5739)

# Exhibit 38



US008084601B2

(12) **United States Patent**  
**Popplewell et al.**

(10) **Patent No.:** **US 8,084,601 B2**  
(45) **Date of Patent:** **Dec. 27, 2011**

(54) **OLIGOMERS**

(75) Inventors: **Linda Popplewell**, Surrey (GB); **Ian Graham**, Cambridge (GB); **John George Dickson**, Surrey (GB)

(73) Assignee: **Royal Holloway and Bedford New College Royal Holloway, University of London**, Surrey (GB)

(\*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **12/556,626**

(22) Filed: **Sep. 10, 2009**

(65) **Prior Publication Data**

US 2010/0168212 A1 Jul. 1, 2010

**Related U.S. Application Data**

(60) Provisional application No. 61/096,073, filed on Sep. 11, 2008, provisional application No. 61/164,978, filed on Mar. 31, 2009.

(51) **Int. Cl.**  
**C07H 21/04** (2006.01)

(52) **U.S. Cl.** ..... **536/24.5**; 536/24.31; 536/24.1;  
514/44

(58) **Field of Classification Search** ..... None  
See application file for complete search history.

(56) **References Cited**

**FOREIGN PATENT DOCUMENTS**

WO	0224906	3/2002
WO	2004083432	9/2004
WO	2006000057	1/2006

**OTHER PUBLICATIONS**

Aartsma-Rus et al. RNA 2007, 13: 1609-1624.\*  
Adams et al. BMC Molecular Biology (2007) "Antisense oligonucleotide induced exon skipping and the dystrophin gene transcript: cocktails and chemistries," 8:57-64.  
Alter et al. Nature Medicine (2006) "Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology," 12:175-177.  
McClorey et al. (2006) "Antisense oligonucleotide-induced exon skipping restores dystrophin expression in vitro in canine model of DMD," 13: 1375-1381.

\* cited by examiner

*Primary Examiner* — Kimberly Chong

(74) *Attorney, Agent, or Firm* — Banner & Witcoff, Ltd.

(57) **ABSTRACT**

Molecules are provided for inducing or facilitating exon skipping in forming spliced mRNA products from pre-mRNA molecules in cells. The molecules may be provided directly as oligonucleotides or expression products of vectors that are administered to a subject. High rates of skipping can be achieved. High rates of skipping reduce the severity of a disease like Duchene Muscular Dystrophy so that the disease is more like Becker Muscular Dystrophy. This is a severe reduction in symptom severity and mortality.

**10 Claims, 11 Drawing Sheets**

FIG. 1

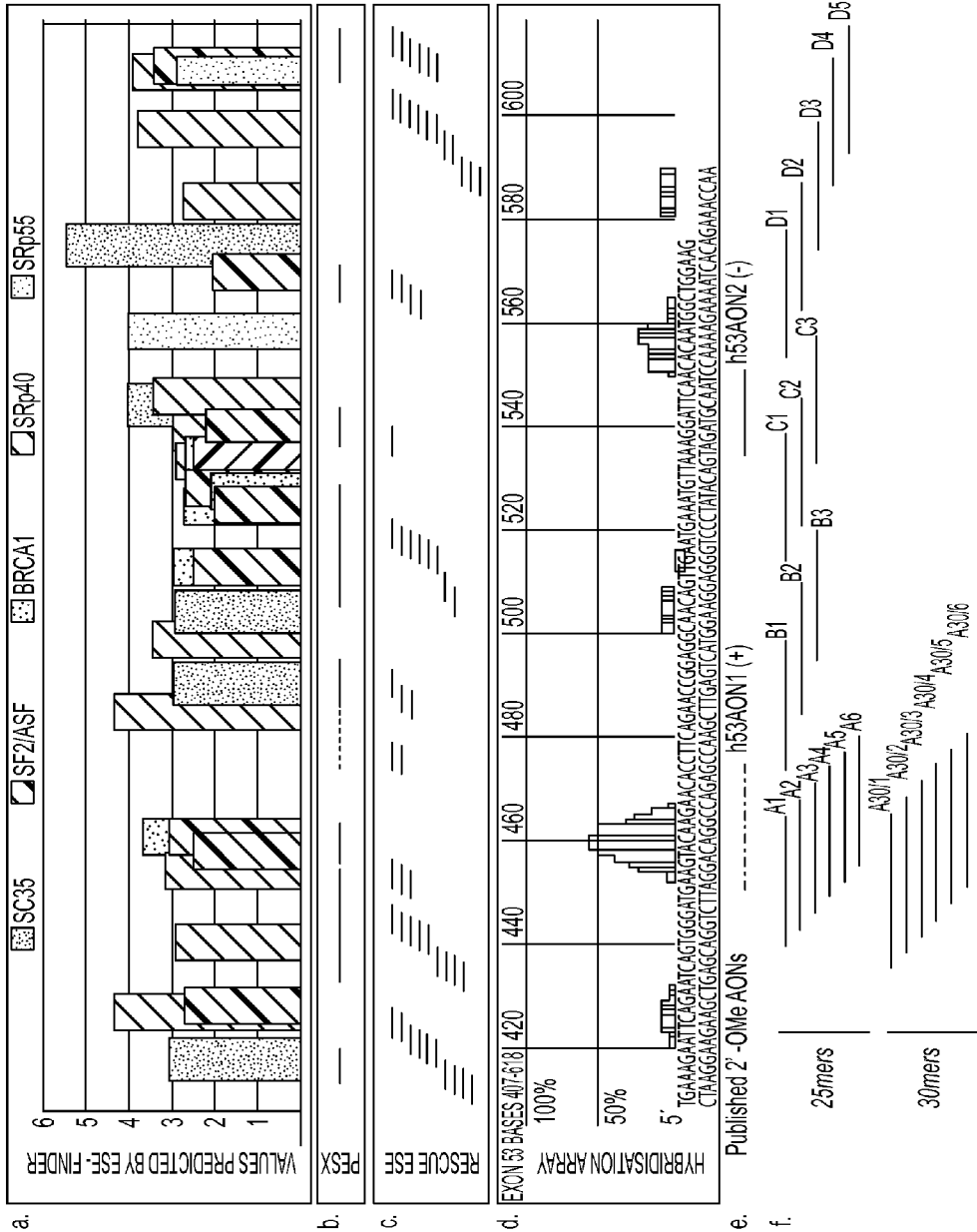


Figure 2

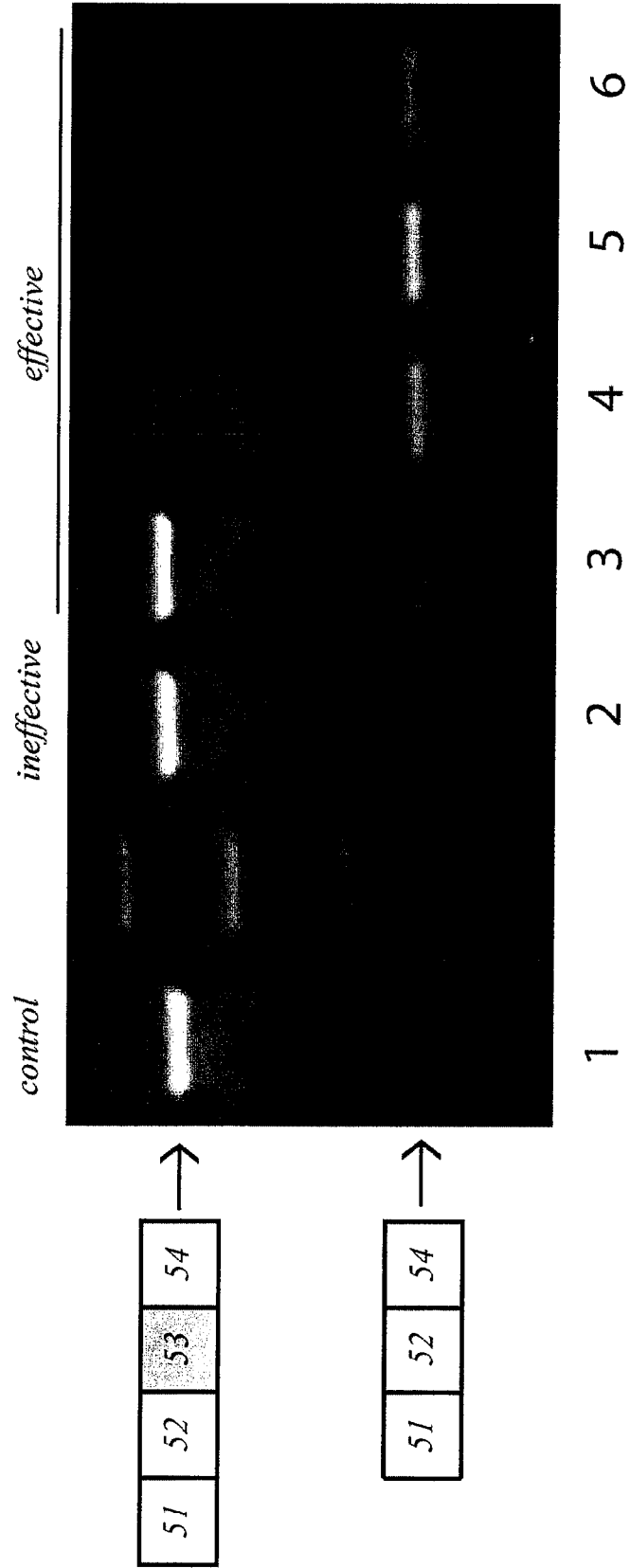


Figure 3

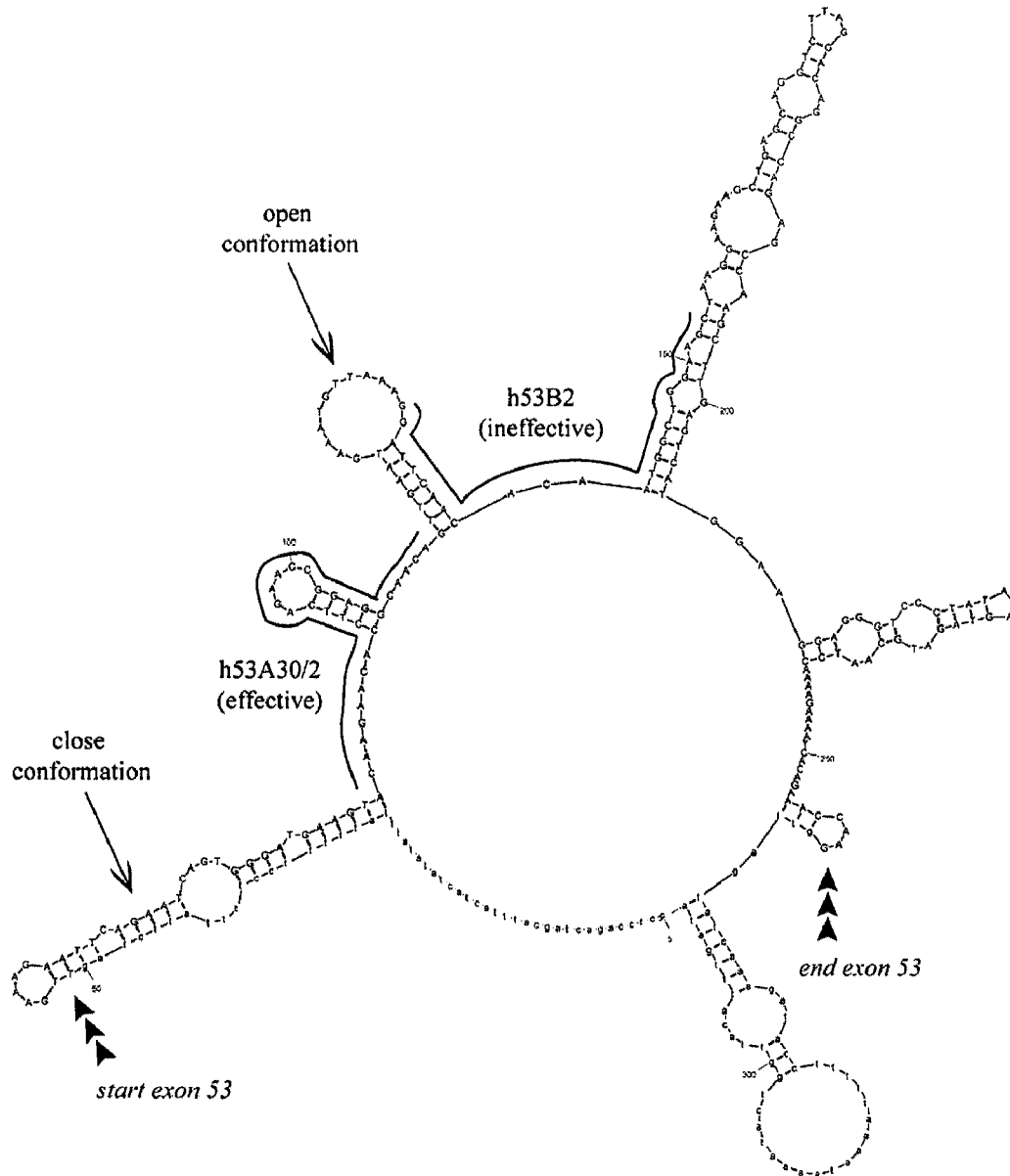


Figure 4

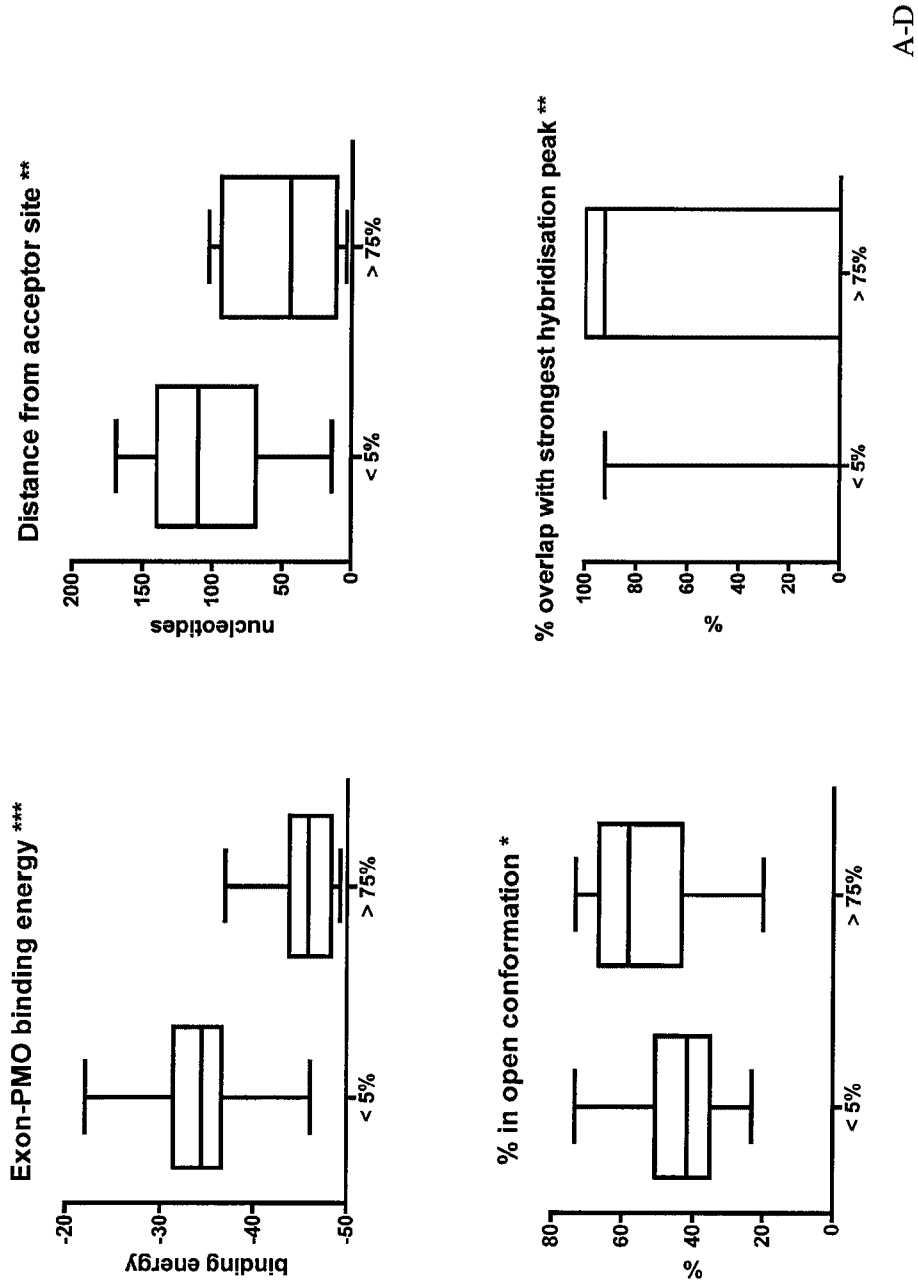


Figure 5A-D

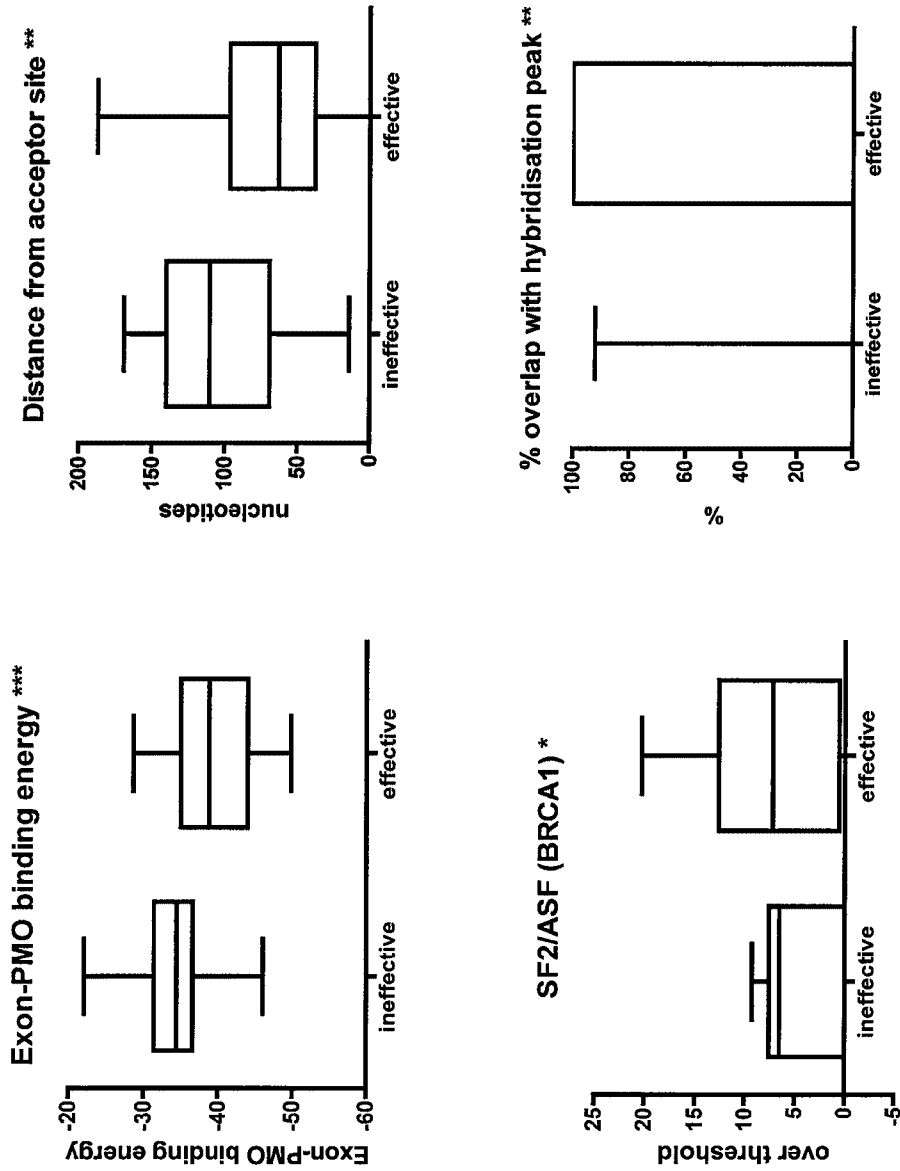
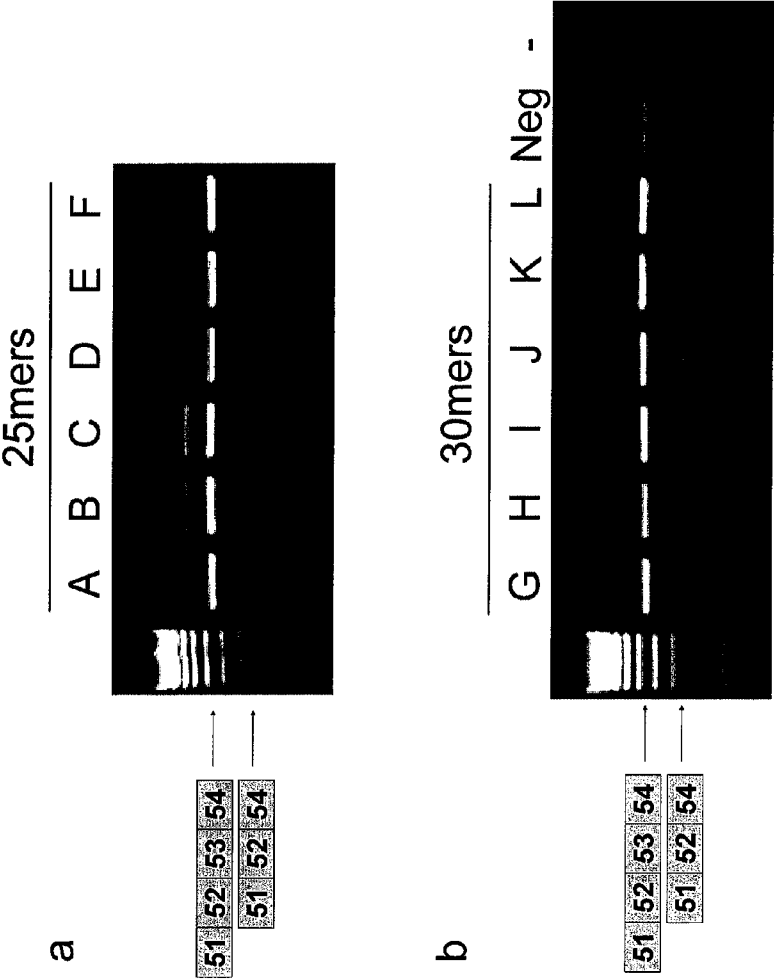


Figure 6



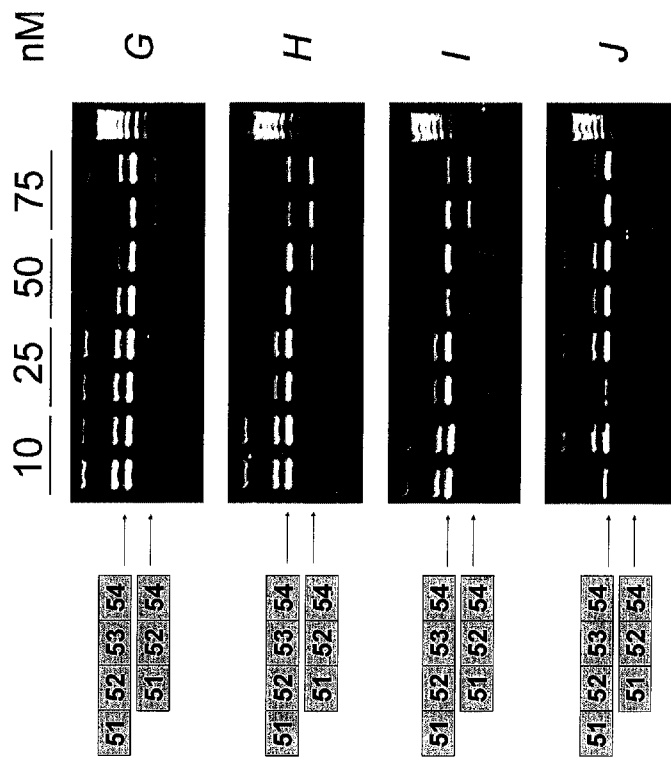
U.S. Patent

Dec. 27, 2011

Sheet 7 of 11

US 8,084,601 B2

Figure 7 a



b

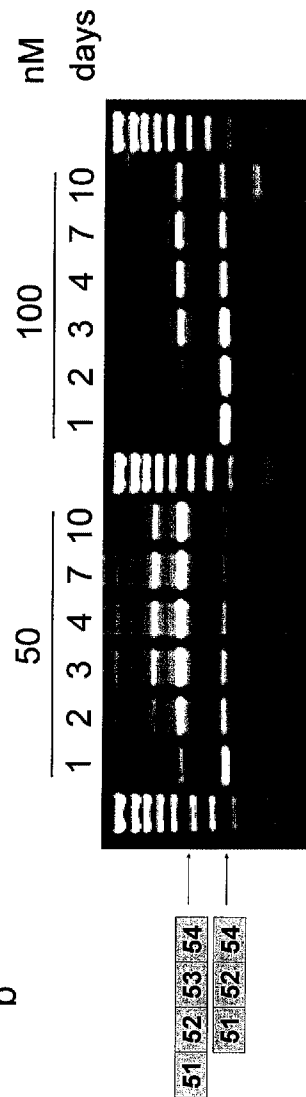


Figure 8

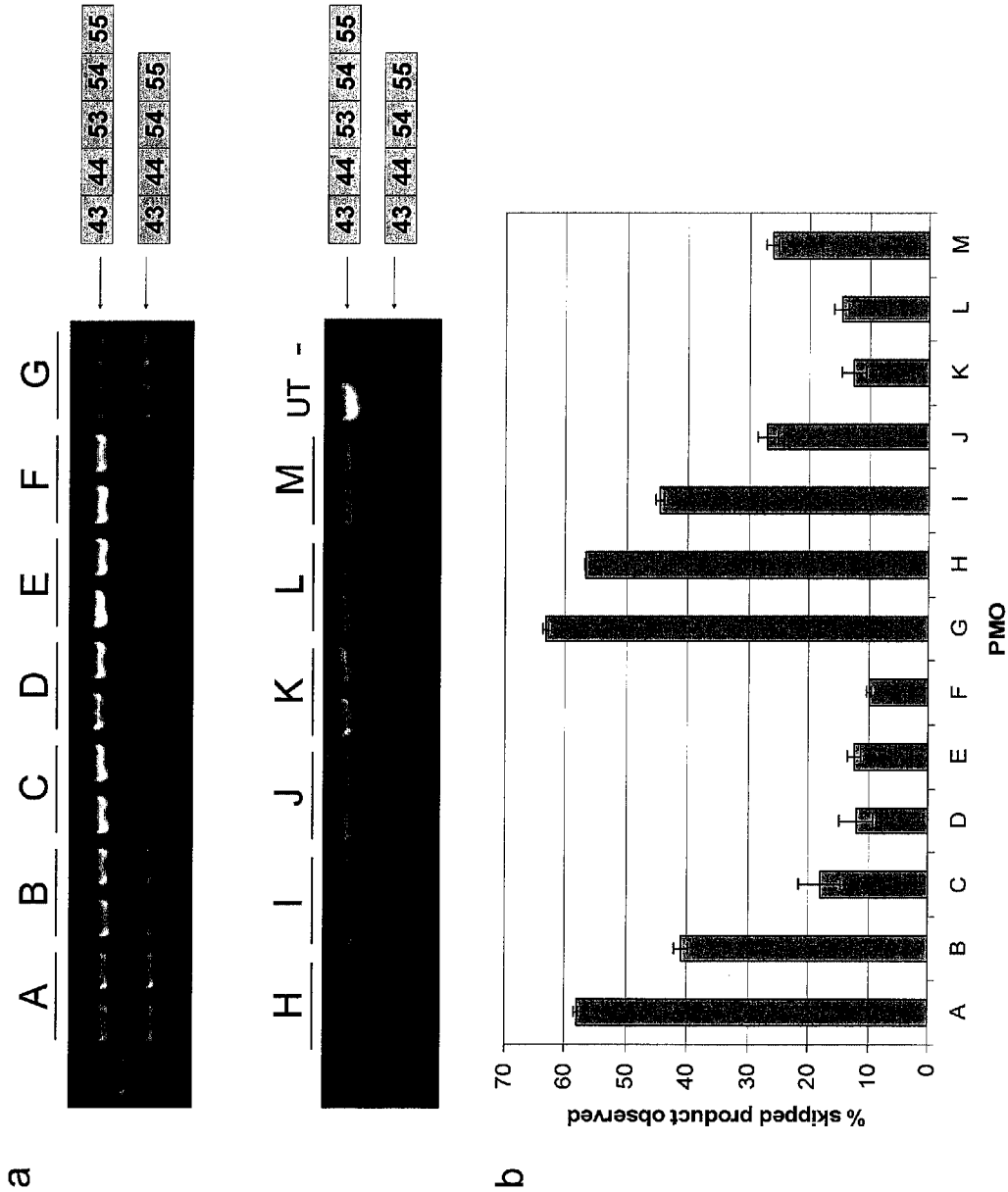


Figure 9

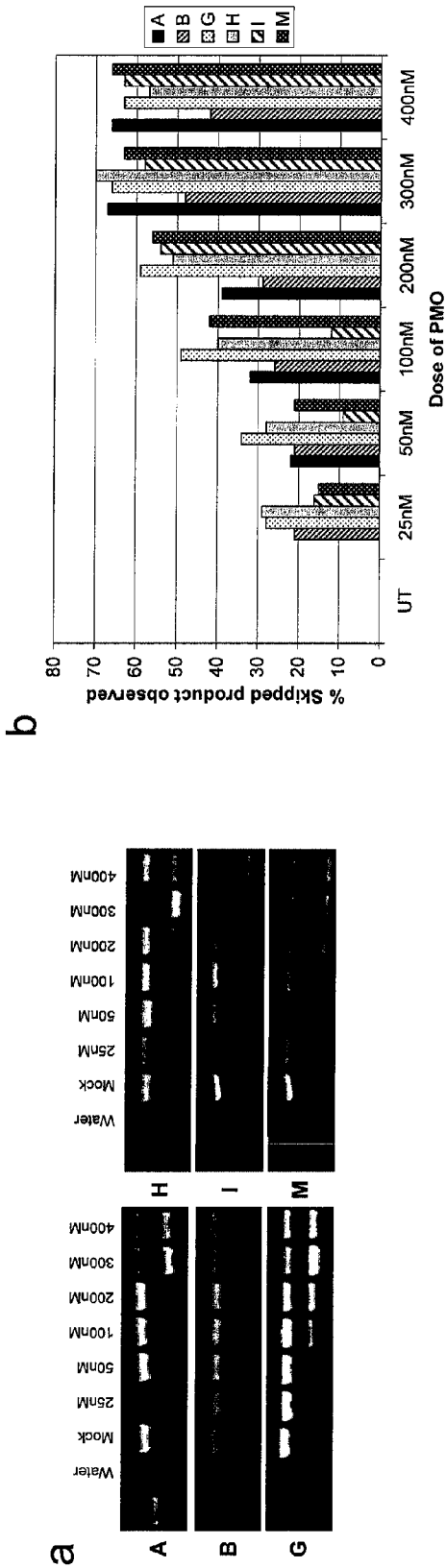
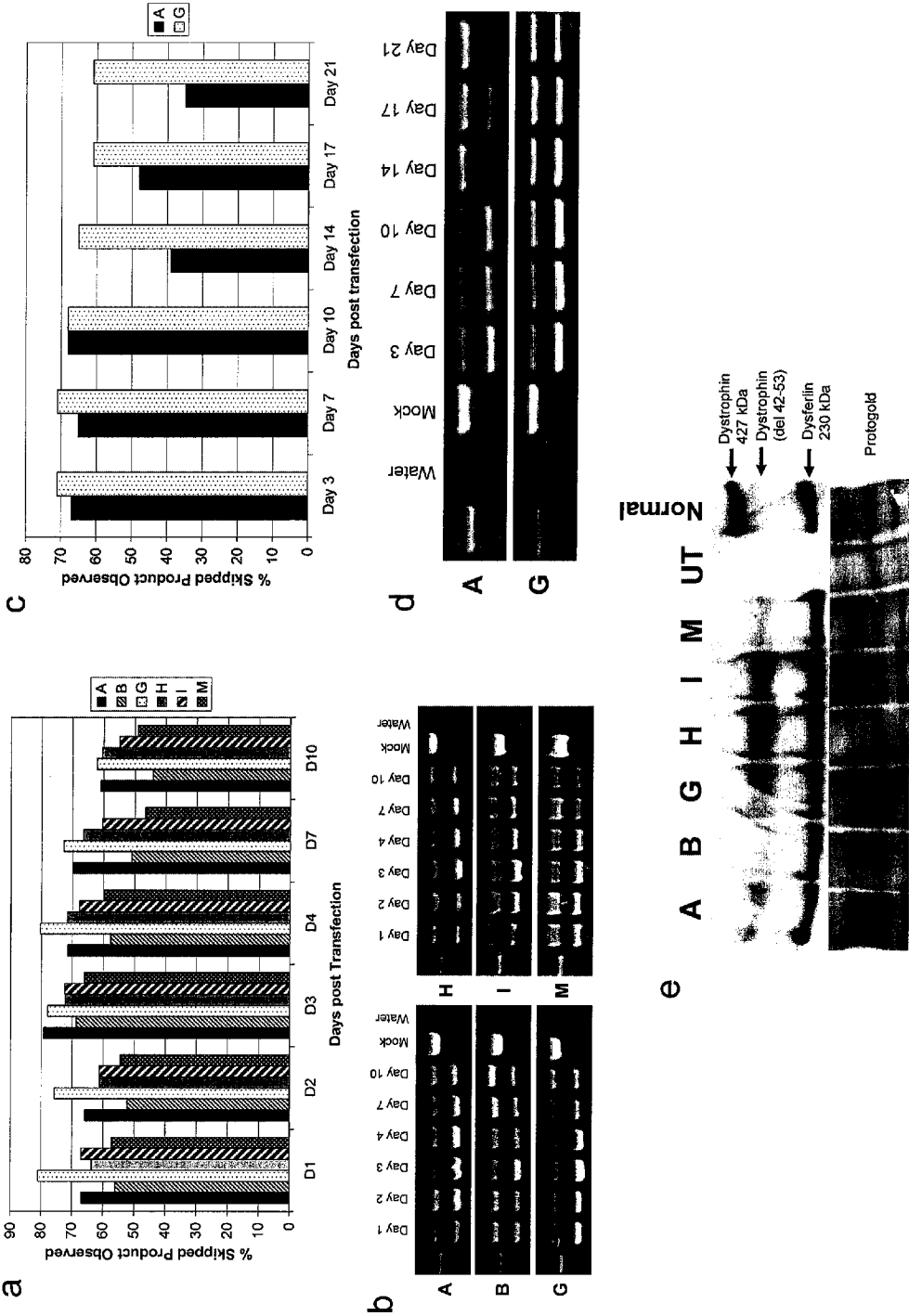


Figure 10



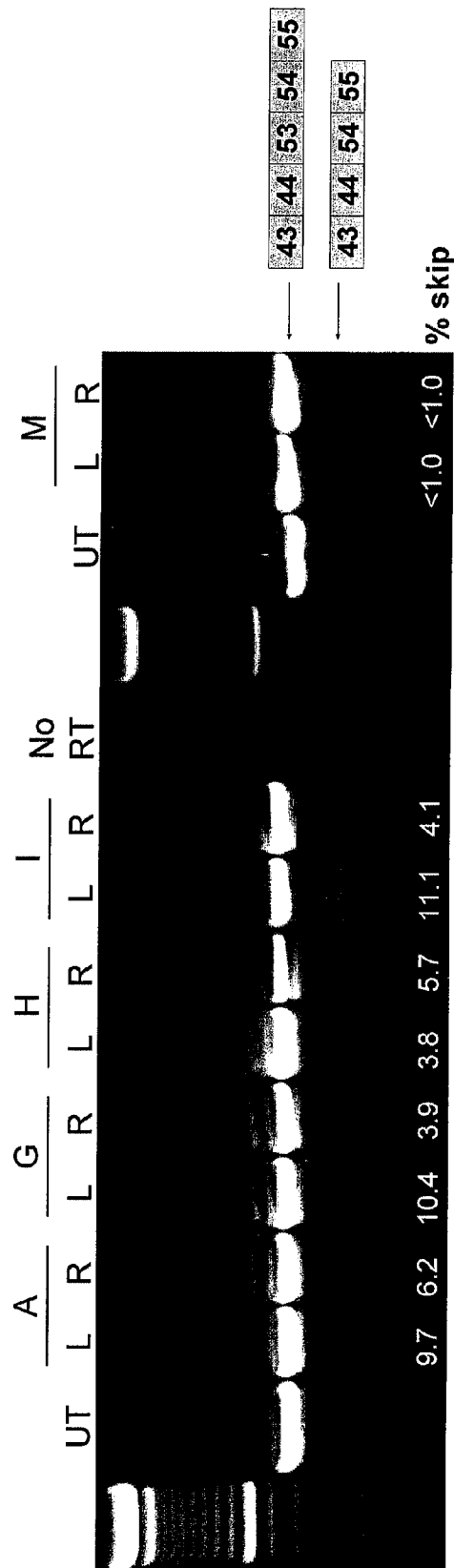
U.S. Patent

Dec. 27, 2011

Sheet 11 of 11

US 8,084,601 B2

Figure 11



US 8,084,601 B2

# 1

## OLIGOMERS

### TECHNICAL FIELD OF THE INVENTION

The present invention relates to molecules which are capable of causing exon skipping and, in particular, relates to molecules which are capable of causing exon skipping in the dystrophin gene.

### BACKGROUND OF THE INVENTION

Duchenne muscular dystrophy (DMD) is a severe X-linked muscle wasting disease, affecting 1:3500 boys. Prognosis is poor: loss of mobility by the age of 12, compromised respiratory and cardiac function by late teens, and probable death by the age of 30. The disease is caused by mutations within the large dystrophin gene, such that the reading frame is disrupted leading to lack of dystrophin protein expression and breakdown of muscle fibre integrity [1]. The dystrophin gene is large, with 79 exons. The most common DMD mutation is genomic deletion of one or more exons, generally centred around hotspots involving exons 44 to 55 and the 5' end of the gene [2]. Mutations of the dystrophin gene that preserve the reading frame result in the milder, non-life threatening Becker muscular dystrophy (BMD).

Exon skipping induced by antisense oligoribonucleotides (AOs), generally based on an RNA backbone, is a future hope as a therapy for DMD in which the effects of mutations in the dystrophin gene can be modulated through a process of targeted exon skipping during the splicing process. The splicing process is directed by complex multi-particle machinery that brings adjacent exon-intron junctions in pre-mRNA into close proximity and performs cleavage of phosphodiester bonds at the ends of the introns with their subsequent reformation between exons that are to be spliced together. This complex and highly precise process is mediated by sequence motifs in the pre-mRNA that are relatively short semi-conserved RNA segments to which bind the various nuclear splicing factors that are then involved in the splicing reactions. By changing the way the splicing machinery reads or recognises the motifs involved in pre-mRNA processing, it is possible to create differentially spliced mRNA molecules.

It has now been recognised that the majority of human genes are alternatively spliced during normal gene expression, although the mechanisms involved have not been identified. Using antisense oligonucleotides, it has been shown that errors and deficiencies in a coded mRNA could be bypassed or removed from the mature gene transcripts. Indeed, by skipping out-of-frame mutations of the dystrophin gene, the reading frame can be restored and a truncated, yet functional, Becker-like dystrophin protein is expressed. Studies in human cells in vitro [3, 4] and in animal models of the disease in vivo [5-9] have proven the principle of exon skipping as a potential therapy for DMD (reviewed in [10]). Initial clinical trials using two different AO chemistries (phosphorodiamidate morpholino oligomer (PMO) and phosphorothioate-linked 2'-O-methyl RNA (2'OMePS)) [11] have recently been performed, with encouraging results. Indisputably impressive restoration of dystrophin expression in the TA muscle of four DMD patients injected with a 2'OMePS AO to exon 51 has been reported by van Deutekom et al. [11].

However, it should be noted that, relative to 2'OMePS AOs, PMOs have been shown to produce more consistent and sustained exon skipping in the mdx mouse model of DMD [12-14; A. Malerba et al, manuscript submitted], in human muscle explants [15], and in dystrophic canine cells in vitro [16].

2

Most importantly, PMOs have excellent safety profiles from clinical and pre-clinical data [17].

The first step to a clinical trial is the choice of the optimal AO target site for skipping of those dystrophin exons most commonly deleted in DMD. In depth analysis of arrays of 2'OMePS AOs have been reported [18, 19], and relationships between skipping bioactivity and AO variables examined.

One problem associated with the prior art is that the antisense oligonucleotides of the prior art do not produce efficient exon skipping. This means that a certain amount of mRNA produced in the splicing process will contain the out-of-frame mutation which leads to protein expression associated with DMD rather than expression of the truncated, yet functional, Becker-like dystrophin protein associated with mRNA in which certain exons have been skipped.

Another problem associated with the prior art is that antisense oligonucleotides have not been developed to all of the exons in the dystrophin gene in which mutations occur in DMD.

An aim of the present invention is to provide molecules which cause efficient exon skipping in selected exons of the dystrophin gene, thus being suitable for use in ameliorating the effects of DMD.

### SUMMARY OF THE INVENTION

The present invention relates to molecules which can bind to pre-mRNA produced from the dystrophin gene and cause a high degree of exon skipping in a particular exon. These molecules can be administered therapeutically.

The present invention provides a molecule for ameliorating DMD, the molecule comprising at least a 25 base length from a base sequence selected from:

- (SEQ ID NO: 1)  
a) XGA AAA CGC CGC CAX XXC XCA ACA GAX CXG;
- (SEQ ID NO: 2)  
b) CAX AAX GAA AAC GCC GCC AXX XCX CAA CAG;
- (SEQ ID NO: 3)  
c) XGX XCA GCX XCX GXX AGC CAC XGA XXA AAX;
- (SEQ ID NO: 4)  
d) CAG XXX GCC GCX GCC CAA XGC CAX CCX GGA;
- (SEQ ID NO: 5)  
e) XXG CCG CXG CCC AAX GCC AXC CXG GAG XXC;
- (SEQ ID NO: 6)  
f) XGC XGC XCX XXX CCA GGX XCA AGX GGG AXA;
- (SEQ ID NO: 7)  
g) CXX XXA GXX GCX GCX CXX XXC CAG GXX CAA;
- (SEQ ID NO: 8)  
h) CXX XXC XXX XAG XXG CXG CXG XXX XCC AGG;
- (SEQ ID NO: 9)  
i) XXA GXX GCX GCX CXX XXC CAG GXX CAA GXG;
- (SEQ ID NO: 10)  
j) CXG XXG CCX CCG GXX CXG AAG GXG XXC XXG;
- (SEQ ID NO: 11)  
k) CAA CXG XXG CCX CCG GXX CXG AAG GXG XXC;
- or
- (SEQ ID NO: 12)  
l) XXG CCX CCG GXX CXG AAG GXG XXC XXG XAC,

wherein the molecule's base sequence can vary from the above sequence at up to two base positions, and wherein the molecule can bind to a target site to cause exon skipping in an exon of the dystrophin gene.

## US 8,084,601 B2

3

The exon of the dystrophin gene is selected from exons 44, 45, 46 or 53. More specifically, the molecule that causes skipping in exon 44 comprises at least a 25 base length from a base sequence selected from:

a) XGA AAA CGC CGC CAX XXC XCA ACA GAX CXG; (SEQ ID NO: 1)  
 b) CAX AAX GAA AAC GCC GCC AXX XCX CAA CAG; (SEQ ID NO: 2)  
 or  
 c) XGX XCA GCX CXG GXX AGC CAC XGA XXA AAX; (SEQ ID NO: 3)

wherein the molecule's sequence can vary from the above sequence at up to two base positions, and wherein the molecule can bind to a target site to cause exon skipping in exon 44 of the dystrophin gene.

The molecule that causes skipping in exon 45 comprises at least a 25 base length from a base sequence selected from:

d) CAG XXX GCC GCX GCC CAA XGC CAX CCX GGA; (SEQ ID NO: 4)  
 or  
 e) XXG CCG CXG CCC AAX GCC AXC CXG GAG XXC; (SEQ ID NO: 5)

wherein the molecule's sequence can vary from the above sequence at up to two base positions, and wherein the molecule can bind to a target site to cause exon skipping in exon 45 of the dystrophin gene.

The molecule that causes skipping in exon 46 comprises at least a 25 base length from a base sequence selected from:

f) XGC XGC XCX XXX CCA GGX XCA AGX GGG AXA; (SEQ ID NO: 6)  
 g) CXX XXA GXX GCX GCX CXX XXC CAG GXX CAA; (SEQ ID NO: 7)  
 h) CXX XXC XXX XAG XXG CXG CXG XXX XCC AGG; (SEQ ID NO: 8)  
 or  
 i) XXA GXX GCX GCX CXX XXC CAG GXX CAA GXG; (SEQ ID NO: 9)

wherein the molecule's sequence can vary from the above sequence at up to two base positions, and wherein the molecule can bind to a target site to cause exon skipping in exon 46 of the dystrophin gene.

The molecule that causes skipping in exon 53 comprises at least a 25 base length from a base sequence selected from:

j) CXG XXG CCX CCG GXX CXG AAG GXG XXC XXG; (SEQ ID NO: 10)  
 k) CAA CXG XXG CCX CCG GXX CXG AAG GXG XXC; (SEQ ID NO: 11)  
 or  
 l) XXG CCX CCG GXX CXG AAG GXG XXC XXG XAC; (SEQ ID NO: 12)

wherein the molecule's sequence can vary from the above sequence at up to two base positions, and wherein the molecule can bind to a target site to cause exon skipping in exon 53 of the dystrophin gene.

4

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a scheme summarizing the tools used in the design of PMOs to exon 53. (a) Results of ESEfinder analysis, showing the location and values above threshold for SF2/ASF, SF2/ASF (BRCA1), SC35, SRp40 and SRp55, shown as grey and black bars, as indicated in the legend above. (b) Output of PESX analysis, showing the location of exonic splicing enhancers as solid lines, and exonic splicing silencer as a dashed line. (c) Rescue ESE analysis for exon 53, showing predicted ESEs by lines, and where they overlap, by a ladder of lines. (d) AccessMapper analysis of in vitro hybridization. Synthetic pre-mRNA (SEQ ID NO: 25) containing exon 53 and surrounding introns was subjected to a hybridization screen against a random hexamer oligonucleotide array, as described in Materials and Methods. Areas of hybridization, suggestive of areas of open conformation, are indicated by peaks on the graph. (e) The position of the target sites of two 2'OMePS AOs studied previously [18] are shown for comparison. (f) The location of the target sites for all the 25mer and 30mer PMOs to exon 53 used in this study are indicated by lines, and numbered according to the scheme used in Table 1, except for exclusion of the prefix "h53".

FIG. 2 shows a comparison of active (effective) and inactive (ineffective) PMOs. RT-PCR analysis of mRNA from normal human skeletal muscle cells treated with PMOs to exon 53 demonstrates a wide variation in the efficiency of exon skipping. Over 75% exon skipping is seen with h53A30/2 (lane 5) and h53A30/3 (lane 6). h53A30/1 (lane 4) produced around 50% skipping, while the 25-mer h53A1 (lane 3) produced just over 10% skipping. In contrast, h53C1 (lane 2) was completely inactive. Lane 1 contains a negative control in which cells were treated with lipofectin but no PMO.

FIG. 3 shows an Mfold secondary structure prediction for exon 53 of the human dystrophin gene. MFOLD analysis [25] was performed using exon 53 plus 50 nt of the upstream and downstream introns (SEQ ID NO:26), and with a maximum base-pairing distance of 100 nt. The intron and exon boundaries are indicated, as are the positions of the target sites of the bioactive PMO h53A30/2 (87.2% skip) and an inactive PMO (h53B2). Examples of open and closed RNA secondary structure are arrowed.

FIG. 4 shows boxplots of parameters significant to strong PMO bioactivity. Comparisons were made between inactive PMOs and those inducing skipping at levels in excess of 75%. Boxplots are shown for parameters which are significant on a Mann-Whitney rank sum test: PMO to target binding energy, distance of the target site from the splice acceptor site, the percentage overlap with areas of open conformation, as predicted by MFOLD software, and the percentage overlap of the target site with the strongest area accessible to binding, as revealed by hexamer hybridization array analysis. Degrees of significance are indicated by asterisks. \*: p<0.05; \*\*: p<0.01; \*\*\*: p<0.001.

FIG. 5 shows boxplots of parameters significantly different between bioactive (effective) and inactive (ineffective) PMOs. Comparisons were made between PMOs determined as bioactive (those that induced skipping at greater than 5%) and those that were not. Boxplots are shown for parameters which are significant from a Mann-Whitney rank sum test: PMO to target binding energy, distance of the target site from the splice acceptor site, the score over threshold for a predicted binding site for the SR protein SF2/ASF, and the percentage overlap of the target site with the strongest area accessible to binding, as revealed by hexamer hybridization

## US 8,084,601 B2

5

array analysis. Degrees of significance are indicated by asterisks. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ .

FIG. 6 shows a comparison of bioactivity of PMOs targeted to exon 53 in normal hSkMCs. Myoblasts were transfected with each of the 25mer (panel a) and 30mer (panel b) PMOs indicated at 500 nM using lipofectin (1:4). RNA was harvested after 24 hours and subjected to nested RT-PCR and products visualised by agarose gel electrophoresis.

FIG. 7 shows low dose efficacy and timecourse of skipping of the most bioactive PMOs in normal hSkMCs. (a) hSkMC myoblasts were transfected with the PMOs indicated over a concentration range of 25 nM to 100 nM using lipofectin (1:4). RNA was harvested after 24 hours and subjected to nested RT-PCR, and products visualised by agarose gel electrophoresis. (b) hSkMC myoblasts were transfected with 100 nM and 500 nM concentrations of PMO-G (+30+59) using lipofectin. RNA was harvested at the timepoints indicated following transfection and subjected to nested RT-PCR, and products visualised by agarose gel electrophoresis. Skipped (248 bp) and unskipped (460 bp) products are shown schematically.

FIG. 8 shows blind comparison of 13 PMO oligonucleotide sequences to skip human exon 53. Myoblasts derived from a DMD patient carrying a deletion of dystrophin exons 45-52 were transfected at 300 nM in duplicate with each of the PMOs by nucleofection. RNA was harvested 3 days following transfection, and amplified by nested RT-PCR. (a) Bars indicate the percentage of exon skipping achieved for each PMO, derived from Image J analysis of the electropherogram of the agarose gel (b). Skipped (477 bp) and unskipped (689 bp) products are shown schematically.

FIG. 9 shows the dose-response of the six best-performing PMOs. (a) Myoblasts derived from a DMD patient carrying a deletion of dystrophin exons 45-52 were transfected with the six best-performing PMOs by nucleofection, at doses ranging from 25 nM to 400 nM. RT-PCR products derived from RNA isolated from cells 3 days post-transfection were separated by agarose gel electrophoresis. (b) The percentage of exon skipping observed is expressed for each concentration of each PMO as a comparison of the percentage OD of skipped and unskipped band, as measured using Image J.

FIG. 10 shows persistence of dystrophin expression in DMD cells following PMO treatment. (a) Myoblasts derived from a DMD patient carrying a deletion of dystrophin exons 45-52 were transfected by nucleofection at 300 nM with each of the six best-performing PMOs, and were cultured for 1 to 10 days before extracting RNA. The percentage of exon skipping was compared using the percentage OD of skipped and unskipped bands, measured using Image J analysis of the agarose gel of the nested RT-PCR products shown in (b). The experiment was repeated, but this time using the two best-performing PMOs from the previous analysis, and continuing the cultures for 21 days post-transfection (c and d). (e) Western blot analysis was performed on total protein extracts from del 45-52 DMD cells 7 days after transfection with the six best PMOs (300 nM). Blots were probed with antibodies to dystrophin, to dysferlin as a muscle-specific loading control, and protogold for total protein loading control. CHQ5B myoblasts, after 7 days of differentiation were used as a positive control for dystrophin protein (normal).

FIG. 11 shows a comparison of most active PMOs in hDMD mice. PMOs were injected in a blind experiment into the gastrocnemius muscle of hDMD mice. RT-PCR analysis of RNA harvested from isolated muscle (L=left, R=right) was performed and products visualised by agarose gel electrophoresis. Quantification of PCR products was performed using a DNA LabChip.

6

## DETAILED DESCRIPTION OF THE INVENTION

Without being restricted to any particular theory, it is thought by the inventors that the binding of the molecules to the dystrophin pre-mRNA interacts with or interferes with the binding of SR proteins to the exon of interest. SR proteins are involved in the slicing process of adjacent exons. Therefore, it is thought that interacting or interfering with the binding of the SR proteins interferes with the splicing machinery resulting in exon skipping.

The base "X" in the above base sequences is defined as being thymine (T) or uracil (U). The presence of either base in the sequence will still allow the molecule to bind to the pre-mRNA of the dystrophin gene as it is a complementary sequence. Therefore, the presence of either base in the molecule will cause exon skipping. The base sequence of the molecule may contain all thymines, all uracils or a combination of the two. One factor that can determine whether X is T or U is the chemistry used to produce the molecule. For example, if the molecule is a phosphorodiamidate morpholino oligonucleotide (PMO), X will be T as this base is used when producing PMOs. Alternatively, if the molecule is a phosphorothioate-linked 2'-O-methyl oligonucleotide (2'OMePS), X will be U as this base is used when producing 2'OMePSs. Preferably, the base "X" is only thymine (T).

The advantage provided by the molecule is that it causes a high level of exon skipping. Preferably, the molecule causes an exon skipping rate of at least 50%, more preferably, at least 60%, even more preferably, at least 70%, more preferably still, at least 76%, more preferably, at least 80%, even more preferably, at least 85%, more preferably still, at least 90%, and most preferably, at least 95%.

The molecule can be any type of molecule as long as it has the selected base sequence and can bind to a target site of the dystrophin pre-mRNA to cause exon skipping. For example, the molecule can be an oligodeoxyribonucleotide, an oligoribonucleotide, a phosphorodiamidate morpholino oligonucleotide (PMO) or a phosphorothioate-linked 2'-O-methyl oligonucleotide (2'OMePS). Preferably, the oligonucleotide is a PMO. The advantage of a PMO is that it has excellent safety profiles and appears to have longer lasting effects in vivo compared to 2'OMePS oligonucleotides. Preferably, the molecule is isolated so that it is free from other compounds or contaminants.

The base sequence of the molecule can vary from the selected sequence at up to two base positions. If the base sequence does vary at two positions, the molecule will still be able to bind to the dystrophin pre-mRNA to cause exon skipping. Preferably, the base sequence of the molecule varies from the selected sequence at one base position and, more preferably, the base sequence does not vary from the selected sequence. The less that the base sequence of the molecule varies from the selected sequence, the more efficiently it binds to the specific exon region in order to cause exon skipping.

The molecule is at least 25 bases in length. Preferably, the molecule is at least 28 bases in length. Preferably, the molecule is no more than 35 bases in length and, more preferably, no more than 32 bases in length. Preferably, the molecule is between 25 and 35 bases in length, more preferably, the molecule is between 28 and 32 bases in length, even more preferably, the molecule is between 29 and 31 bases in length, and most preferably, the molecule is 30 bases in length. It has been found that a molecule which is 30 bases in length causes efficient exon skipping. If the molecule is longer than 35 bases in length, the specificity of the binding to the specific

## US 8,084,601 B2

7

exon region is reduced. If the molecule is less than 25 bases in length, the exon skipping efficiency is reduced.

The molecule may be conjugated to or complexed with various entities. For example, the molecule may be conjugated to or complexed with a targeting protein in order to target the molecule to muscle tissue. Alternatively, the molecule may be complexed with or conjugated to a drug or another compound for treating DMD. If the molecule is conjugated to an entity, it may be conjugated directly or via a linker. In one embodiment, a plurality of molecules directed to exon skipping in different exons may be conjugated to or complexed with a single entity. Alternatively, a plurality of molecules directed to exon skipping in the same exon may be conjugated to or complexed with a single entity. For example, an arginine-rich cell penetrating peptide (CPP) can be conjugated to or complexed with the molecule. In particular, (R-Ahx-R)(4)AhxB can be used, where Ahx is 6-amino-hexanoic acid and B is beta-alanine [35], or alternatively (RXRRBR)2XB can be used [36]. These entities have been complexed to known dystrophin exon-skipping molecules which have shown sustained skipping of dystrophin exons in vitro and in vivo.

In another aspect, the present invention provides a vector for ameliorating DMD, the vector encoding a molecule of the invention, wherein expression of the vector in a human cell causes the molecule to be expressed. For example, it is possible to express antisense sequences in the form of a gene, which can thus be delivered on a vector. One way to do this would be to modify the sequence of a U7 snRNA gene to include an antisense sequence according to the invention. The U7 gene, complete with its own promoter sequences, can be delivered on an adeno-associated virus (AAV) vector, to induce bodywide exon skipping. Similar methods to achieve exon skipping, by using a vector encoding a molecule of the invention, would be apparent to one skilled in the art.

The present invention also provides a pharmaceutical composition for ameliorating DMD, the composition comprising a molecule as described above or a vector as described above and any pharmaceutically acceptable carrier, adjuvant or vehicle. Pharmaceutical compositions of this invention comprise any molecule of the present invention, and pharmaceutically acceptable salts, esters, salts of such esters, or any other compound which, upon administration to a human, is capable of providing (directly or indirectly) the biologically active molecule thereof, with any pharmaceutically acceptable carrier, adjuvant or vehicle. Pharmaceutically acceptable carriers, adjuvants and vehicles that may be used in the pharmaceutical compositions of this invention include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat.

The pharmaceutical compositions of this invention may be administered orally, parenterally, by inhalation spray, topically, rectally, nasally, buccally, vaginally, intradermally or via an implanted reservoir. Oral administration or administration by injection is preferred. The pharmaceutical compositions of this invention may contain any conventional non-toxic pharmaceutically-acceptable carriers, adjuvants or

8

vehicles. The term parenteral as used herein includes subcutaneous, intracutaneous, intravenous, intramuscular, intra-articular, intrasynovial, intrasternal, intrathecal, intralesional and intracranial injection or infusion techniques. Preferably, the route of administration is by injection, more preferably, the route of administration is intramuscular, intravenous or subcutaneous injection and most preferably, the route of administration is intravenous or subcutaneous injection.

The pharmaceutical compositions may be in the form of a sterile injectable preparation, for example, as a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to techniques known in the art using suitable dispersing or wetting agents (such as, for example, Tween 80) and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are mannitol, water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono- or diglycerides. Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions may also contain a long-chain alcohol diluent, dispersant or similar alcohol.

The pharmaceutical compositions of this invention may be orally administered in any orally acceptable dosage form including, but not limited to, capsules, tablets, and aqueous suspensions and solutions. In the case of tablets for oral use, carriers which are commonly used include lactose and corn starch. Lubricating agents, such as magnesium stearate, are also typically added. For oral administration in a capsule form, useful diluents include lactose and dried corn starch. When aqueous suspensions are administered orally, the active ingredient is combined with emulsifying and suspending agents. If desired, certain sweetening and/or flavouring and/or colouring agents may be added.

The pharmaceutical compositions of this invention may also be administered in the form of suppositories for rectal administration. These compositions can be prepared by mixing a compound of this invention with a suitable non-irritating excipient which is solid at room temperature but liquid at the rectal temperature and therefore will melt in the rectum to release the active components. Such materials include, but are not limited to, cocoa butter, beeswax and polyethylene glycols.

Topical administration of the pharmaceutical compositions of this invention is especially useful when the desired treatment involves areas or organs readily accessible by topical application. For application topically to the skin, the pharmaceutical composition should be formulated with a suitable ointment containing the active components suspended or dissolved in a carrier. Carriers for topical administration of the compounds of this invention include, but are not limited to, mineral oil, liquid petroleum, white petroleum, propylene glycol, polyoxyethylene polyoxypropylene compound, emulsifying wax and water. Alternatively, the pharmaceutical composition can be formulated with a suitable lotion or cream containing the active compound suspended or dissolved in a carrier. Suitable carriers include, but are not limited to, mineral oil, sorbitan monostearate, polysorbate 60, cetyl esters wax, cetaryl alcohol, 2-octyldodecanol, benzyl alcohol and water. The pharmaceutical compositions of this invention

## US 8,084,601 B2

9

may also be topically applied to the lower intestinal tract by rectal suppository formulation or in a suitable enema formulation. Topically-transdermal patches are also included in this invention.

The pharmaceutical compositions of this invention may be administered by nasal aerosol or inhalation. Such compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other solubilizing or dispersing agents known in the art.

In one embodiment, the pharmaceutical composition may comprise a plurality of molecules of the invention, each molecule directed to exon skipping in a different exon. Alternatively, the pharmaceutical composition may comprise a plurality of molecules of the invention, each molecule directed to exon skipping in the same exon.

In another embodiment, the pharmaceutical composition may comprise a plurality of vectors of the invention, each vector encoding a molecule directed to exon skipping in a different exon. Alternatively, the pharmaceutical composition may comprise a plurality of vectors of the invention, each vector encoding a molecule directed to exon skipping in the same exon.

In yet another embodiment, the pharmaceutical composition may comprise a molecule and a vector, wherein the molecule and the molecule encoded by the vector are directed to exon skipping in the same or different exons.

The present invention also provides a molecule of the invention for use in therapy.

Further, the present invention provides a molecule of the invention for use in the amelioration of DMD.

The molecules of the present invention cause exon skipping in the dystrophin pre-mRNA. This causes a truncated but functional dystrophin protein to be expressed which results in a syndrome similar to Becker muscular dystrophy (BMD). Therefore, the symptoms of DMD will not be completely treated but will be ameliorated so that they are potentially no longer life threatening.

The present invention also provides a method of ameliorating DMD in a human patient, the method comprising administering a therapeutically effective amount of the molecule of the invention to the patient.

The particular molecule that is administered to the patient will depend on the location of the mutation or mutations present in the dystrophin gene of the patient. The majority of patients have deletions of one or more exons of the dystrophin gene. For example, if a patient is missing exon 44, the process of joining exon 43 to exon 45 will destroy the protein, thus causing DMD. If exon 45 is skipped using a molecule of the invention, the joining of exon 43 to exon 46 will restore the protein. Similarly, a patient with a deletion of exon 45 can be treated with a molecule to skip either exon 44 or exon 46. Further, a patient with a deletion of exons 45 to 52 inclusive (a large portion of the gene), would respond to skipping of exon 53.

In another aspect, the invention provides a kit for the amelioration of DMD in a patient, the kit comprising a molecule of the invention and instructions for its use. In one embodiment, the kit may contain a plurality of molecules for use in causing exon skipping in the same exon or a plurality of exons.

## EXAMPLES

## Example 1

Here, the first detailed study of the role that AO target site variables have on the efficacy of PMOs to induce skipping is

10

reported. The results reported here should have an impact on the initial planning and design of AOs for future potential clinical trials.

## Materials and Methods

## Hybridization Analyses

Templates for the production of synthetic pre-mRNAs for exons 44, 45, 46, 51, and 53 of the human dystrophin gene (DMD gene) were generated by PCR amplification from genomic clones of the exons, together with approximately 500 nt of upstream and downstream introns. PCR primers incorporated T7 RNA polymerase promoter sequences, such that pre-mRNAs could be produced by in vitro transcription. Pre-mRNAs were then subjected to a hybridization screen against a spotted array of all 4096 possible hexanucleotide sequences (Access Array 4000; Nyrion Ltd, Edinburgh UK). Binding of the pre-mRNA to specific spots on the array was detected by reverse transcriptase-mediated incorporation of biotinylated nucleotides by primer extension, followed by fluorescent labelling. Scanning of the arrays followed by software analysis enabled sequences within the exons that were accessible to binding to the hexamer array to be identified. Using a hybridization assay, binding accessibility of each exon was analysed and hybridization peak identified by AccessMapper software (Nyrion Ltd) (see FIG. 1d).

## AO Design

Overlapping AOs were designed to exons 44, 45, 46, 51, and 53 of the human DMD gene using the following information: putative SR protein binding domains as predicted by ESEfinder [20, 21], Rescue ESE [24] and PESX [22, 23] analyses of exon sequence; sequences accessible to binding as determined by hybridization analyses (Nyrion); previously published work [18, 19].

All AOs were synthesized as phosphorodiamidate morpholino oligos (PMOs) by Gene Tools LLC (Philomath Oreg., USA). To facilitate transfection of these uncharged oligonucleotides into cultured cells, the PMOs were hybridized to phosphorothioate-capped oligodeoxynucleotide leashes, as described by Gebiski et al., [12], and stored at 4° C.

The sequences of some of these PMOs were as follows:

(SEQ ID NO: 13)  
H44A30/1 -  
TGA AAA CGC CGC CAT TTC TCA ACA GAT CTG;

(SEQ ID NO: 14)  
H44A30/2 -  
CAT AAT GAA AAC GCC GCC ATT TCT CAA CAG;

(SEQ ID NO: 15)  
H44AB30/2 -  
TGT TCA GCT TCT GTT AGC CAC TGA TTA AAT;

(SEQ ID NO: 16)  
H45A30/2 -  
CAG TTT GCC GCT GCC CAA TGC CAT CCT GGA;

(SEQ ID NO: 17)  
H45A30/1 -  
TTG CCG CTG CCC AAT GCC ATC CTG GAG TTC;

(SEQ ID NO: 18)  
H46A30/2 -  
TGC TGC TCT TTT CCA GGT TCA AGT GGG ATA;

(SEQ ID NO: 19)  
H46A30/4 -  
CTT TTA GTT GCT GCT CTT TTC CAG GTT CAA;

(SEQ ID NO: 20)  
H46A30/5 -  
CTT TTC TTT TAG TTG CTG CTC TTT TCC AGG;

## US 8,084,601 B2

11

-continued

(SEQ ID NO: 21)  
H46A30/3 -  
TTA GTT GCT GCT CTT TTC CAG GTT CAA GTG;  
  
(SEQ ID NO: 22)  
H53A30/2 -  
CTG TTG CCT CCG GTT CTG AAG GTG TTC TTG;  
  
(SEQ ID NO: 23)  
H53A30/3 -  
CAA CTG TTG CCT CCG GTT CTG AAG GTG TTC;  
  
(SEQ ID NO: 24)  
H53A30/1 -  
TTG CCT CCG GTT CTG AAG GTG TTC TTG TAC.

## Cell Culture and AO Transfection

Normal human primary skeletal muscle cells (TCS Cellworks, Buckingham, UK) were seeded in 6-well plates coated with 0.1 mg/ml ECM Gel (Sigma-Aldrich, Poole, UK), and grown in supplemented muscle cell growth medium (Promocell, Heidelberg, Germany). Cultures were switched to supplemented muscle cell differentiation medium (Promocell) when myoblasts fused to form visible myotubes (elongated cells containing multiple nuclei and myofibrils). Transfection of PMOs was then performed using the transfection reagent Lipofectin (Invitrogen, Paisley, UK) at a ratio of 4  $\mu$ l of Lipofectin per  $\mu$ g of PMO (with a range of PMO concentrations tested from 50 to 500 nM, equivalent to approximately 0.5 to 5  $\mu$ g) for 4 hrs, according to the manufacturer's instructions. All transfections were performed in triplicate in at least two different experiments.

## RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction Analysis

Typically 24 h after transfection, RNA was extracted from the cells using the QIAshredder/RNeasy system (Qiagen, Crawley, UK) and ~200 ng RNA subjected to RT-PCR with DMD exon-specific primers using the GeneScript kit (Genesys, Camberley, UK). From this 20 cycle reaction, an aliquot was used as a template for a second nested PCR consisting of 25 cycles. PCR products were analysed on 1.5% agarose gels in Tris-borate/EDTA buffer. Skipping efficiencies were determined by quantification of the PCR products by densitometry using GeneTools software (Syngene, Cambridge, UK).

## Statistical Analysis

The non-parametric Mann-Whitney rank sum test was used to identify whether parameters for effective PMOs were significantly different to those for ineffective PMOs. Where data was calculated to fit a normal distribution, the more powerful two-tailed Student's t-test was performed instead. Correlations were generated using the Spearman rank-order test. To determine the strength of the combined significant parameters/design tools to design effective PMOs, linear discriminant analysis was used [34], with the Ida function from the MASS package, using "effective" or "ineffective" as the two prior probabilities. The Ida function produces posterior probabilities for the two classes (effective and ineffective) for each PMO by leave-one-out classification.

## Results

## PMO Design and Analysis of Bioactivity

A unique set of 66 PMOs has been designed to target exons 44, 45, 46, 51, and 53 of the human gene for dystrophin. The design process for exon 53 is depicted in FIG. 1, and has also been performed for the other four exons (data not shown). The exon sequence was analysed for the presence of exonic splicing enhancers (ESE) and exonic splicing suppressors or silencers (ESS) and the outputs aligned for the three available algorithms, ESEfinder (FIG. 1a) [20, 21], PESX (FIG. 1b) [22, 23], and Rescue ESE (FIG. 1c) [24]. Hybridization array

12

analysis was also performed for each exon in vitro, as described in Materials and Methods. The peaks shown in FIG. 1d indicate areas of the exon that are in a conformation able to hybridize to the array, and which may consequently prove more accessible to antisense AOs. The coincidence of ESEs, as predicted by two or more algorithms, and hybridization peaks determined experimentally, was used to design arrays of 25mer and, subsequently, 30mer PMOs, the positions of which are shown in FIG. 1f. The binding sites for 2'OMePS AOs described previously [18] are shown for comparison (FIG. 1e).

Each PMO was tested in primary cultures of human skeletal muscle, in triplicate, in at least two experiments, and over a range of concentrations from 50 nM to 500 nM. Their bioactivity was determined by RT-PCR analysis, which showed a wide variation in the level of exon skipping induced (FIG. 2, and data not shown), ranging from 0% for h53C1 (FIG. 1f and FIG. 2, lane 2) to 80% for h53A30/3 (FIG. 1f and FIG. 2, lane 6). Sequencing of the PCR products verified accurate skipping of the targeted exon (data not shown). The activity of each PMO at the stated optimal concentration is summarized in Table 1. Bioactivity is expressed as a percentage of the skipped amplicon relative to total PCR product, as assessed by densitometry. Specific, consistent and sustained exon skipping was evident for 44 of the 66 PMOs tested.

## In Silico Analysis of PMOs

We then performed a retrospective in silico analysis of the characteristics of all 66 PMOs tested in this study, with respect to PMO length, the distance of the PMO target site from the splice donor and acceptor sites, PMO-to-target binding energy and PMO-to-PMO binding energy, as calculated using RNAstructure2.2 software for the equivalent RNA-RNA interaction, and percentage GC content of the PMO, the results of which are summarized in Table 1. Also shown in Table 1 is the percentage overlap of each PMO target site with sequences shown to be accessible to binding, as determined experimentally by the hexamer hybridization array analysis. The relationship of PMO target site and RNA secondary structure was also examined using the program MFOLD [25] (FIG. 3 and data not shown), with the percentage overlap of PMO target site with sequence predicted to be in open conformation by MFOLD analysis given in Table 1. ESEfinder and SSF (<http://www.umd.be/SSF/>) software analysis of exon sequences revealed the positions of putative SR protein binding motifs (SF2/ASF (by two algorithms), SC35, SRp40, SRp55, Tra2 $\beta$  and 9G8). The highest score over threshold for each SR protein is given for each PMO in the columns on the right of Table 1. Also shown is the degree of overlap of each PMO target site with the ESE and ESS regions predicted by Rescue ESE and PESX.

## Statistical Analysis of Design Parameters in Relation to PMO Bioactivity

For this statistical analysis, bioactive PMOs are considered to be those which produce over 5% skipping, while those that produce less than 5% skipping are considered inactive. For each of the parameters listed in Table 1, comparison was made between bioactive and inactive PMOs using the non-parametric Mann-Whitney rank sum test, or, when it was statistically valid to do so, the parametric Student's t-test (two-tailed). The significant parameters are listed in Table 2. Considering the data as a whole, the variable which showed the highest significance to PMO bioactivity was the binding energy of the PMO to the exon ( $p=0.001$ ); the most bioactive exons are predicted to bind better to their target sites. Those PMOs that overlap with peaks identified by the experimental hybridization array analysis are not significantly more active than those that do not ( $p=0.056$ ), but when only the strongest

peak for each exon is considered, this parameter becomes highly significant ( $p=0.003$ ). Distance of the PMO target site to the splice acceptor site of the exon was also highly significant ( $p=0.004$ ), with PMOs whose target site were closer to the acceptor site being more active. PMOs whose target sites showed coincidence with binding motifs for the SR protein SF2/ASF (as defined by the BRCA1 algorithm of Smith et al. [21]) produced significantly greater skipping ( $p=0.026$ ). PMO length is also a significant parameter ( $p=0.017$ ), with longer PMOs being more effective at inducing skipping. Box-plots of the significant variables identified here are shown in FIG. 5. None of the other variables considered in this study were shown to have any significance to AO bioactivity.

To ascertain which parameters/design tools are the most powerful, we also used the Mann-Whitney rank sum test to compare the most active PMOs (i.e. those that induce greater than 75% skipping of the target exon) to those that were inactive (i.e. those that produce less than 5% skipping). Box-plots of the significant variables for this comparison are shown in FIG. 4. There is strong significance of overlap of the PMO target site with the strongest hybridization peak for each exon ( $p=0.002$ ); more of the most bioactive PMOs had their target sites coincident with sequences accessible to binding than those that were inactive. This is reinforced by the observation that the target sites of PMOs that produced over 75% skipping significantly overlapped more RNA that was in open conformation, relative to inactive PMOs ( $p=0.025$ ). Stronger binding between the PMO and its target exon, PMO length, and proximity of the target to the acceptor site are also significant parameters when comparing the most and least effective reagents.

Spearman's rank order correlation was used to establish potential relationships between design parameters and skipping bioactivity using the entire set of PMOs. This shows a strong correlation between skipping bioactivity and PMO-target binding energy ( $r_s=-0.618$ ,  $p=0$ ), percentage open conformation ( $r_s=0.275$ ,  $p=0.0259$ ), PMO length ( $r_s=0.545$ ,  $p=0$ ), distance from the splice acceptor site ( $r_s=-0.421$ ,  $p=0$ ), percentage overlap with the strongest hybridization peak ( $r_s=0.46$ ,  $p=0$ ), and overlap with an ESS sequence, as predicted by PESX ( $r_s=0.261$ ,  $p=0.0348$ ).

#### Linear Discriminant Analysis

This analysis was performed on all possible combinations of length, overlap with the SF2/ASF (BRCA1) motif, percentage overlap with areas of open conformation, percentage overlap with hybridization peak and PMO-target binding energy, i.e. PMO parameters and design tools that showed significance or borderline significance. Using length, SF2/ASF (BRCA1) motif and hybridization peak data, nine of the inactive PMOs were classified as bioactive and four bioactive PMOs were classified as inactive (Table 3). These four misclassified PMOs were 25mers to exon 46, three of which have borderline bioactivity, i.e. produce just 10% skipping, while the fourth produces about 20% skipping. Taken overall, this equates to 80% of the PMOs being predicted correctly when assessed according to their length, SF2/ASF (BRCA1) overlap and hybridization peak overlap. This would suggest that these parameters have the potential to be effective design tools, with four out of every five PMOs designed to have these three properties likely to be bioactive. In line with this, there was a distinct trend for PMOs being correctly assigned as bioactive with increased skipping bioactivity (see Table 3). Indeed, the PMOs with greatest bioactivity were all 30mers (10/10), bound to their target with a high binding energy of below  $-43.0$  kD (9/10), overlapped by over 50% with areas of

open conformation (7/10), overlapped with SF2/ASF (BRCA1) peak (8/10), and overlapped with a hybridization peak (7/10).

#### Discussion

Clinical studies using AOs to skip exon 51 to correct DMD deletions are progressing well [11; F. Muntoni, Principal Investigator of MDEX Consortium, personal communication]. However since the mutations that cause DMD are so diverse, skipping of exon 51 would have the potential to treat just 24.6% of DMD patients on the Leiden DMD database [26]. It is therefore imperative that pre-clinical optimization of AO target sequence and chemistry is continually studied and improved. This study has examined the significance of design parameters for PMO-induced skipping of exons 44, 45, 46, 51, and 53, which would have the potential to treat, respectively, 11.5%, 15.8%, 8.4%, 24.6% and 13.5% of DMD patients in the Leiden database [26; A. Aartsma-Rus, personal communication].

Specific skipping was observed for the five DMD exons studied here, with two-thirds of the PMOs tested being bioactive. This proportion of bioactive AOs within a cohort has been reported previously [18, 19], but we have induced high-level (i.e. greater than 75%) skipping in four of the five exons tested, some of which are achievable at relatively low doses of oligomer. The exception is exon 51, published previously [4], achieving a maximal skipping of 26%. The work of Wilton et al [19] demonstrated that only exons 51 and 53 can be skipped with high efficiency ( $>30\%$  by their definition), and that exons 44, 45 and 46 are less "skippable" (less than 30% skipping). Furthermore, Aartsma-Rus et al [18] showed oligomers capable of high-level skipping (greater than a mere 25%) for only exons 44, 46 and 51.

We provide here direct evidence that AO bioactivity shows a significant association with accessibility of its target site to binding. This is the first study to assess sequences practically within the pre-mRNA that are accessible to binding and then use them as an aid to AO design. The data we show underline the value of the hybridization analysis in determining what are likely to be the most bioactive oligomers (i.e. those that produce greater than 75% skipping). As an example, if we look at the data for oligomers developed for exon 45 [18], we see that there is only one moderately effective (5-25%) reagent for this otherwise unskippable exon. This oligomer is the only one of the six tested that overlaps with the strongest peak in our hybridization analysis. The partial nature of this overlap, combined with the short length of the oligomer, is likely to contribute to its relative weakness compared to the PMOs we have developed here. In general, the 2'OMePS AOs displaying the highest bioactivity in the work of Aartsma-Rus et al. [18] and Wilton et al. [19], show some degree of overlap with the hybridization peaks that we have defined here for exons 45, 46 and 53.

Ease of skipping of certain DMD exons has been seen elsewhere [18] and may be related to other factors affecting splicing, including strength of splice donor and acceptor sites and branchpoint, and the size of upstream and downstream introns, which may affect the order in which exons are spliced together. There is the potential of using a cocktail of AOs to induce greater skipping of the more difficult to skip exons [27, 28].

Accessibility of the AO to its target site depends directly on the secondary structure of the pre-mRNA, which has a major role in determining AO bioactivity in cells. A study in which the structure around an AO target site was changed revealed that AOs were unable to invade very stable stem-loop structures and their antisense activity was inhibited, but generally showed good activity when impeded by little local structure

[29]. Overlap of PMO target sites with open conformations in the folded RNA showed a weak association with PMO bioactivity, which was more obvious when only the stronger PMOs were considered in the statistical analysis. It is also possible that there is selective pressure for SR binding sites to be located preferentially on these open secondary structures. The presumption is that binding of bioactive PMOs to their target sites sterically block the binding of important factors involved in RNA processing, resulting in exon skipping.

One of the PMO parameters with high significance was length; 30mer PMOs were far superior to their 25mer counterparts. The influence of 2'OMePS AO length on bioactivity has been reported elsewhere [30] and such an observation for PMO-induced skipping of exon 51 has been reported previously by us [4]. The more persistent action of longer PMOs would have important cost and dose implications in the choice of AO for clinical trials. Longer AOs are likely to sterically block more of the regions that interact with the splicing machinery, but in general terms, the energy of binding of the longer PMO to its target would be increased, which we showed to be the most significant parameter in AO design. The strong significance of the binding energy of PMO-target complexes (i.e., free energy of AO-target compared to free energy of the target) and PMO length to bioactivity suggests that PMO bioactivity depends on stability of the PMO-target complex, and implies that bioactive PMOs act by interference of target RNA folding. Computational analysis revealed that the thermodynamics of binding of active PMOs to their target site had a dramatic effect on the secondary folded structure of the RNA (data not shown). It is likely that these changes in secondary structure would have a profound effect on the binding of SR proteins to the RNA, thereby disrupting splicing, and exon skipping would ensue.

Overlap of a PMO target site with a binding site motif for the SR protein SF2/ASF (BRCA1), as predicted by ESEfinder, showed a significant association to PMO bioactivity. This partly confirms the work of Aartsma-Rus et al. [18], who observed marginally significantly higher ESEfinder values for SF2/ASF and SC35 motifs for effective AOs when compared to inactive AOs. SC35 and SF2/ASF motifs are the two most abundant proteins assessed by ESEfinder. The reason why we do not see any significance of overlap with SC35 motif to PMO bioactivity may be due to the difference in AO chemistry used, and the number of AOs assessed. However Aartsma-Rus et al. [18] did note that not every bioactive AO has a high value for any of the SR protein binding motifs, and some inactive AOs have high values. The

apparent weakness and unreliability of SR protein binding motifs as design tools for AOs may be a reflection of the lack of precision of the predictive software used. Overlap of PMO target site with exonic splicing silencers appears to show a correlation with bioactivity in Spearman's rank order test analysis. Such a correlation would be counter-intuitive and the true significance questionable. Again the strength of the predictive software used may be in doubt. It should be noted that the software programmes used predict SR binding motifs on the linear exon sequence. The availability of these predicted motifs to bind SR proteins, or for binding PMOs to disrupt the binding of these proteins, is directly related to the folding of the pre-mRNA. The discrepancy in the relative significance of secondary RNA structure and SR protein binding motifs may be due to active PMOs disrupting SR protein binding, not sterically but indirectly, by altering the secondary pre-mRNA structure. A very recent study has shown the importance of co-transcriptional pre-mRNA folding in determining the accessibility of AO target sites and their effective bioactivity, and showed a direct correlation between AO bioactivity and potential interaction with pre-mRNA [31].

It has been previously reported that ESE sites located within 70 nucleotides of a splice site are more active than ESE sites beyond this distance [32]. Our results partially support this; PMOs with their target site closer to the splice acceptor site are significantly more bioactive. However distance of the PMO target site to the splice donor site showed no statistical significance to bioactivity. This bias has been previously reported for the analyses of 2'OMePS AOs [18, 19], and may be related to the demonstration, by Patzel et al. [33], of the importance of an unstructured 5' end of RNA in the initiation of hybridization of oligonucleotide binding. This would suggest that targeting any significant parameters located in the 5' part of an exon may increase the probability of designing a bioactive AO.

In conclusion, our findings show that no single design tool is likely to be sufficient in isolation to allow the design of a bioactive AO, and empirical analysis is still required. However this study has highlighted the potential of using a combination of significant PMO parameters/design tools as a powerful aid in the design of bioactive PMOs. Linear discriminant analysis revealed that using the parameters of PMO length, overlap with SF2/ASF (BRCA1) motif and hexamer array hybridization data in combination would have an 80% chance of designing a bioactive PMO, which is an exciting and surprising finding, and should be exploited in further studies.

TABLE 1

Table 1: Table summarizing the characteristics of PMOs used											
PMO	Targeted	Optimol	%			Exon-PMO	PMO-PMO	Ends in open		Distance from	
	exon	conc.	Skip <sup>a</sup>	Length	% GC	binding energy	binding energy	% open <sup>b</sup>	loops <sup>b</sup>	donor	acceptor
h53B1	53	500	0	25	28	-22.1	-12.1	53.3	1	119	68
h53C1	53	500	0	25	48	-32.4	-9.8	46.7	2	79	108
h53C2	53	500	0	25	56	-31.3	-12.7	33.3	1	72	115
h53C3	53	500	0	25	60	-34.6	-13.7	26.7	1	60	127
h53D1	53	500	0	25	52	-34.1	-13.4	30	1	39	148
h45A30/4	45	500	0	30	43	-35.2	-7.5	40	1	43	93
h45A30/6	45	500	0	30	53	-42.4	-26.9	46.7	2	9	137
h46A10	46	500	0	25	40	-35.3	-1.7	23.3	1	63	60
h46A30/6	53	500	0	30	40	-42.1	-10.1	56.7	0	5	113
h53D2	46	500	0.1	25	48	-36.5	-14.5	40	2	30	157
h46A5	53	500	0.2	25	36	-33.9	-7.9	53.3	0	10	113
h53A6	53	500	0.3	25	48	-35.3	-8.5	43.3	2	138	49
h53B2	53	500	0.6	25	48	-30.1	-11.3	23.3	1	108	79
h46A11	46	500	0.6	25	20	-24.5	-1.5	43.3	0	0	143

## US 8,084,601 B2

17

18

TABLE 1-continued

Table 1: Table summarizing the characteristics of PMOs used											
h46A30/8	46	500	1.5	30	30	-34.2	-1.8	46.7	0	0	136
h45A30/7	45	500	1.6	30	50	-46.1	-4.7	73.3	0	0	158
h45A30/8	45	500	1.6	30	40	-39.3	-13.7	53.3	1	76	70
h53A3	53	500	2	25	56	-36.7	-13.7	36.7	0	147	40
h46A9	46	500	2.1	25	28	-31.5	-7.6	36.7	1	109	14
h53B3	53	500	3	25	48	-34.5	-5.5	48	2	98	89
h53D3	53	500	3.7	25	36	-34.3	-11.2	40	1	18	169
h44B30/8	44	500	4.6	30	37	-28.3	-23.5	40	1	34	84
h44B30/4	44	50	5	30	43	-38.2	-14.6	40	0	54	64
h46A6	46	100	5.4	25	36	-31.5	-8	46.7	1	0	123
h46A8	46	500	5.4	25	32	-28.6	0	20	1	76	47
h45A30/3	45	500	6.3	30	40	-35.5	-11.8	60	1	108	38
h53D5	53	500	7.9	25	36	-31.5	-3.3	66.7	1	0	187
h46A1	46	100	8.3	25	48	-35.7	-11.9	53.3	1	38	85
h53A5	53	250	9	25	48	-35.5	-8.5	43.3	2	141	46
h46A7	46	500	9.1	25	32	-34.8	-5.6	36.7	1	123	0
h53A30/5	53	100	9.4	30	47	-42.4	-11.3	46.7	1	141	41
h53A2	53	100	9.7	25	56	-36.1	-17.4	46.7	1	150	37
h53A4	53	500	10.5	25	48	-34.3	-8.5	20	0	144	43
h45A30/5	45	500	11.2	30	63	-44	-21.1	26.7	0	17	129
h53D4	53	500	12.3	25	32	-30.9	-9.2	63.3	1	6	181
h53A1	53	100	12.7	25	52	-38.6	-17.4	50	2	153	34
A25	51	250	14.9	25	36	-29.3	-11.6	66.7	2	146	62
h46A2	46	500	15.6	25	44	-31.2	-10.6	56.7	1	33	90
h46A30/7	46	500	18.5	30	30	-34.2	-6.2	53.3	1	0	141
h46A4	46	100	21.2	25	44	-39.9	-6.3	56.7	2	20	103
h44C30/2	44	50	22	30	33	-38	-7.4	36.7	1	7	111
h44B30/7	44	100	26	30	37	-33.9	-10.9	26.7	1	39	79
h51A	51	500	26.3	30	43	-40.3	-15	70	1	137	65
h44B30/6	44	500	32.5	30	37	-34.6	-9.6	30	2	44	74
h44C30/3	44	500	35	30	33	-38.9	-13.8	30	1	2	116
h44B30/1	44	100	35	30	33	-35.2	-7.1	66.7	1	69	49
h53A30/6	53	500	35.9	30	47	-42.3	-8.5	56.7	1	338	44
h53A30/4	53	100	38.6	30	50	-43.4	-17.4	43.3	1	144	38
h44C30/1	44	100	42	30	37	-41.1	-10.4	50	1	12	106
h46A3	46	100	49.7	25	48	-43.1	-5.2	56.7	2	28	95
h44A30/3	44	250	52.1	30	37	-42.5	-8.6	56.7	1	99	19
h53A30/1	53	100	52.4	30	50	-48.1	-17.4	56.7	1	153	29
h44B30/3	44	500	61	30	43	-35.4	-11.4	30	0	59	59
h44B30/5	44	500	63.3	30	40	-35.9	-14.6	30	1	49	69
h45A30/1	45	500	64.5	30	60	-49.7	-11	36.7	1	146	0
h46A30/3	46	500	74.6	30	43	-49.8	-6.1	73.3	2	23	95
h46A30/1	46	500	75.6	30	47	-43.5	-12.3	63.3	0	33	85
h46A30/5	46	500	76.7	30	40	-49.2	-6.3	70	1	15	103
h53A30/3	53	100	80.1	30	53	-44.6	-17.4	53.3	1	147	35
h44B30/2	44	500	80.5	30	37	-36.9	-10.7	50	1	64	54
h53A30/2	53	100	87.2	30	53	-45.1	-17.4	63.3	1	150	32
h46A30/4	46	500	87.3	30	40	-47.5	-6.3	73.3	2	20	98
h46A30/2	46	500	87.9	30	47	-49.1	-13.4	63.3	2	28	90
h45A30/2	45	500	91.4	30	60	-46.6	-13	20	1	142	4
h44A30/2	44	500	95	30	43	-44	-8.6	40	0	104	14
h44A30/1	44	250	97	30	47	-47.5	-11.2	46.7	1	109	9

PMO	% overlap with hybrid. peak	% Rescue ESE sites	% overlap with Rescue ESE	% overlap with		ESE finder values over threshold <sup>c</sup>						
				PESE	PESS	SF2/ASF	BRCA1	SC35	SRp40	SRp55	Tra2B	9G8
h53B1	0	5	56	40	40	0	9.26	3.62	10.66	0	5.06	1.1
h53C1	0	6	52	72	0	4.19	6.72	0	2.04	0	24.04	28.68
h53C2	0	1	24	60	0	4.19	6.72	10.2	4.38	0	0	8.28
h53C3	0	1	24	32	0	3.49	6.41	10.2	4.38	6.86	0	14.18
h53D1	0	4	40	32	0	0.52	0	18.68	0	6.86	0	12.71
h45A30/4	100	4	40	0	0	6.29	4.8	5.9	17.91	0	18.18	8.14
h45A30/6	100	4	40	0	0	11.64	7.34	5.04	1.38	0	7.25	16.53
h46A10	0	7	60	48	8	2.21	0	2.7	2.88	0	5.11	23.85
h46A30/6	0	7	40	50	0	0	0	0	5.09	0	24.04	6.94
h53D2	0	6	44	32	0	0.52	1.8	18.68	0.42	0	0	12.71
h46A5	0	7	48	44	0	0	0	0	5.09	0	24.04	6.94
h53A6	92	2	36	28	32	6.58	7.26	0	0	0	7.25	11.9
h53B2	0	5	60	60	0	0	9.26	3.62	4.73	0	5.06	8.28
h46A11	0	2	36	12	52	0	0	0	1.02	0	0	2.04
h46A30/8	0	1	27	27	43	0	0	0	1.02	0	0	2.04
h45A30/7	100	9	47	0	0	6.34	7.34	0	0.6	0	18.18	8.14
h45A30/8	100	4	47	0	0	0	0	5.9	2.4	0	18.18	17.14
h53A3	0	3	32	60	0	6.58	7.26	0	3.12	0	7.25	11.9
h46A9	0	8	48	25	0	0	7.87	0	0	0	24.04	7.14
h53B3	0	8	72	64	0	3.49	9.26	3.44	4.73	0	24.04	28.68

## US 8,084,601 B2

19

20

TABLE 1-continued

Table 1: Table summarizing the characteristics of PMOs used												
h53D3	0	9	64	0	0	0	1.8	0	6.95	0	24.04	10.49
h44B30/8	0	7	57	27	13	2.85	8.64	7.06	1.38	0	10.92	19.02
h44B30/4	0	8	47	37	27	1.98	8.64	6.14	10.12	0	7.25	8.28
h46A6	0	7	72	64	0	0	0	0	5.09	0	24.04	6.94
h46A8	0	5	56	24	60	2.21	0	3.56	2.88	0	0	23.68
h45A30/3	100	9	87	30	0	0	6.18	3.07	4.73	0.45	24.04	28.68
h53D5	0	14	92	44	0	8.5	11.95	0	7.67	0.33	24.04	7.14
h46A1	100	3	20	40	0	2.62	20.26	6.63	6.17	0	0	5.12
h53A5	100	3	36	36	20	6.58	7.26	0	3.12	0	7.25	11.9
h46A7	0	9	64	44	0	0	0	6.02	4.2	0	24.04	28.68
h53A30/5	100	5	47	47	17	6.58	7.26	0	3.12	0	7.25	11.9
h53A2	100	4	32	72	0	6.58	7.26	0	3.12	0	7.25	19.02
h53A4	100	4	28	48	8	6.58	7.26	0	3.12	0	7.25	11.9
h45A30/5	100	2	23	0	0	11.64	13.49	5.04	1.38	0	7.25	16.53
h53D4	0	16	96	24	0	8.5	11.95	0	7.67	0.33	24.04	7.14
h53A1	92	7	56	84	0	6.58	7.26	0	3.12	0	24.04	19.02
A25	0	1	24	12	32	1.22	13.72	0	0	0	0	0
h46A2	100	5	40	40	0	2.62	20.26	6.63	6.17	0.0	13.11	5.12
h46A30/7	0	2	20	10	43	0	0	0	1.02	0	0	2.1
h46A4	46	8	60	40	0	0	0	0	5.09	0	24.04	6.94
h44C30/2	0	3	33	10	63	0.52	5.72	0	0	0	9.46	5.6
h44B30/7	0	6	40	30	27	2.85	8.64	7.06	1.38	0	10.92	19.02
h51A	0	2	40	3	27	1.22	13.72	0	0	0	0	4.45
h44B30/6	0	8	37	20	27	2.85	8.64	0	1.92	0	10.92	19.02
h44C30/3	0	2	33	0	63	0	0	0	6.44	0	9.46	5.6
h44B30/1	0	6	67	33	30	0	0	6.14	10.12	0	10.92	8.28
h53A30/6	100	5	48	37	27	6.58	7.26	0	3.12	0	7.25	11.9
h53A30/4	100	4	43	57	7	6.58	7.26	0	3.12	0	7.25	11.9
h44C30/1	0	3	43	27	63	0.52	5.72	7.06	0	0	9.46	5.6
h46A3	100	5	40	40	0	2.62	20.26	6.03	6.17	0	13.11	5.12
h44A30/3	0	3	23	0	77	0	13.26	0	0	0	0	11.3
h53A30/1	92	9	60	86	0	6.58	7.26	0	3.12	0	24.04	19.02
h44B30/3	0	5	47	37	33	0	0	6.14	10.12	0	7.25	8.28
h44B30/5	0	10	63	37	27	1.98	8.64	6.14	1.92	0	10.92	19.02
h45A30/1	100	2	0	0	6.7	3.43	8.64	5.16	3.54	3.57	0	20.56
h46A30/3	100	5	40	13	0	0	0.57	0	6.17	0	13.11	5.12
h46A30/1	100	5	33	33	0	2.62	20.26	6.63	6.17	0	13.11	5.12
h46A30/5	46	12	67	50	0	0	0	0	5.09	0	24.04	6.94
h53A30/3	100	6	43	67	0	6.58	7.26	0	3.12	0	24.04	19.02
h44B30/2	0	5	50	37	37	0	0	6.14	10.12	0	7.25	8.28
h53A30/2	100	8	53	77	0	6.58	7.26	0	3.12	0	24.04	19.02
h46A30/4	85	8	50	43	0	0	0.57	0	5.09	0	24.04	5.12
h46A30/2	100	5	33	33	0	2.62	20.26	6.63	6.17	0	13.11	5.12
h45A30/2	100	0	0	0	20	3.43	10.41	5.16	3.54	3.57	0	20.56
h44A30/2	0	3	27	0	63	0	13.26	0	0	0	0	11.3
h44A30/1	0	4	43	0	47	0	13.26	0	2.76	0	0	11.3

PMOs are ranked in order of efficacy and characteristics of the PMOs and their target sites listed.

<sup>a</sup>calculated as % skipped amplicon relative to total amplicon (i.e. skipped plus full length) as assessed by densitometric analysis of RT-PCR gels.

<sup>b</sup>calculated as % on PMO target site in open structures on predicted RNA secondary structure obtained using MFOLD analysis. The position of the PMO target sites relative to open loops in the RNA secondary structure is listed (0 = no ends in open loops, 1 = one end in an open loop, 2 = both ends in open loops).

<sup>c</sup>In analyses, SR binding sites were predicted using splice sequence finder (<http://www.umd.be/SSF/>) software. Values above threshold are given for PMOs whose target sites cover 50% or more of potential SR binding sites for SF2/ASF, BRCA1, SC35, SRp40, SRp55, Tra2β and 9G8.

TABLE 2

Table 2: The correlation of significant design parameters and PMO target site properties to skipping efficacy							
Comparison	PMO-target binding energy	% open conformation	Length	Distance from acceptor site	% overlap with hybridisation peak	% overlap with strongest hybrid. peak	% overlap with BRCA1 motif
Ineffective vs Effective	0.001	0.094	0.017	0.004	0.056	0.003	0.026
Ineffective vs 5-25% skip	0.534	0.288	1	0.163	0.107	0.034	0.205
Ineffective vs 25-50% skip	0.02	0.316	0.014	0.067	0.614	0.195	0.079
Ineffective vs 50-75% skip	0.002	0.438	0.012	0.005	0.352	0.084	0.341
Ineffective vs 75-100% skip	<0.001	0.025	0.002	0.003	0.045	0.002	0.091
Ineffective vs >50% skip	<0.001	0.052	<0.001	<0.001	0.05	0.005	0.046
Spearman's correlation coefficient	-0.618	0.275	0.545	-0.421	0.258	0.46	0.261
Spearman's p value	0	0.0259	0	0	0.0363	0	0.0341

To establish the significance of design parameters and PMO target site properties to bioactivity, Mann-Whitney rank sum test analysis was performed for each, comparing ineffective (inactive) PMOs to the different groups of PMOs, subdivided (in the column headed "Comparison") according to bioactivity (efficacy). Criteria with p-values less than 0.05 in one or more comparisons are shown. The correlation of these variables to bioactivity is confirmed by Spearman rank order test analysis, for which Spearman correlation coefficients and p-values are given.

US 8,084,601 B2

21

TABLE 3

Table 3: Linear discriminant analysis of effective and ineffective PMOs					
Group	Classification		Total	Error rate	Average score
	Effective	Ineffective			
Effective	40	4	44	0.09	0.741
Ineffective	9	13	22	0.41	0.512
0-5% skip	9	13	22	0.41	0.512
5-25% skip	16	4	20	0.2	0.621
25-50% skip	9	0	9	0	0.806
50-75% skip	6	0	6	0	0.827
75-100% skip	10	0	10	0	0.857

Linear discriminant analysis [34] was used to predict the classification of PMOs on the basis of their PMO-target binding energy, overlap of PMO target site with a hybridization peak, and overlap of PMO target site with an ASF/SF2 (BRCA1) motif. PMOs have been grouped on the basis of their experimental bioactivity ("Group" column), and PMOs within each group predicted as "Effective" (bioactive) or "Ineffective" (inactive), as indicated by the column headings, according to the parameters used in the statistical analysis. The error rate for wrongly classifying a PMO, and the average score are given for each subgroup of PMO.

### Example 2

Here, the inventors show the comparative analysis of a series of PMOs targeted to exon 53, skipping of which would have the potential to treat a further 8% of DMD patients with genomic deletions on the Leiden database compared to skipping of exon 51 which has the potential to treat 13% of DMD patients [37]. An array of overlapping PMOs were designed for the targeting of exon 53 as described previously [38]. These were all tested initially in normal human skeletal muscle cells (hSkMCs), since these are more readily available than patient cells. PMOs that showed greatest skipping efficacy were further tested in cells from a DMD patient with a relevant deletion (del 45-52). The PMOs with greatest efficacy, in terms of concentration and stability, were evaluated by performing dose-response and time-course studies. Findings from these experiments were supported by in vivo studies in a mouse model transgenic for the entire human dystrophin locus [8]. Collectively, this work suggests that one particular PMO (A, h53A30/1, +30+59) produced the most robust skipping of exon 53, and should be considered the sequence of choice for any upcoming PMO clinical trial.

#### Materials and Methods

##### AO Design

Twenty-three overlapping AOs to exon 53 were designed as described above in Example 1.

##### Cell Culture and AO Transfection

Transfections were performed in two centres (Royal Holloway, London UK (RHUL) and UCL Institute of Child Health, London UK (UCL)) and by two different methods (liposome-carrier of leashed PMOs in normal cells (RHUL), and by nucleofection of naked PMOs in patient cells (UCL)). AOs were transfected into normal human primary muscle cells (TCS Cellworks, Buckingham, UK) and into patient primary skeletal muscle cultures obtained from muscle biopsies taken at the Dubowitz Neuromuscular Unit, UCL Institute of Child Health (London, UK), with the approval of the institutional ethics committee. Normal hSkMCs were cultured and transfected with leashed PMOs, using 1:4 lipofectin, as described previously [4]. To minimize any influence of leash design on PMO uptake and subsequent bioactivity, the DNA sequences in the leashes were of the same length (17mers for the 25mer PMOs or 20mers for the 30mer PMOs) and were completely complementary to the 3'-most 17 or 25 nt of each PMO. The phosphorothioate caps of 5 nt at each end were not complementary to the PMOs, and had the same sequences for every leash.

22

##### DMD Patient Primary Myoblast Culture

Skeletal muscle biopsy samples were taken from a diagnostic biopsy of the quadriceps from a DMD patient with a deletion of exons 45-52. Informed consent was obtained before any processing of samples. Muscle precursor cells were prepared from the biopsy sample by sharp dissection into 1 mm<sup>3</sup> pieces and disaggregated in solution containing HEPES (7.2 mg/ml), NaCl (7.6 mg/ml), KCl (0.224 mg/ml) Glucose (2 mg/ml) Phenol Red (1.1 µg/ml) 0.05% Trypsin-0.02% EDTA (Invitrogen, Paisley, UK) in distilled water, three times at 37° C. for 15 minutes in Wheaton flasks with vigorous stirring. Isolated cells were plated in non-coated plastic flasks and cultured in Skeletal Muscle Growth Media (Promocell, Heidelberg, Germany) supplemented with 10% Foetal Bovine Serum (PAA Laboratories, Yeovil, UK), 4 mM L-glutamine and 5 µg/ml gentamycin (Sigma-Aldrich, Poole, UK) at 37° C. in 5% CO<sub>2</sub>.

##### Nucleofection of DMD Primary Myoblasts

Between 2×10<sup>5</sup> and 1×10<sup>6</sup> cells/ml were pelleted and resuspended in 100 µl of solution V (Amaxa Biosystems, Cologne, Germany). The appropriate PMO to skip exon 53 was added to the cuvette provided, sufficient to give the concentrations described, followed by the cell suspension, and nucleofected using the Amaxa nucleofector 2, program B32. 500 µl of media was added to the cuvette immediately following nucleofection. This suspension was transferred to a 6 well plate in differentiation medium. Nucleofected cells were maintained in differentiation media for 3-21 days post treatment before extraction of RNA or protein.

##### Lactate Dehydrogenase Cytotoxicity Assay

A sample of medium was taken 24 hours post-transfection to assess cytotoxicity by release of lactate dehydrogenase (LDH) into the medium, using the LDH Cytotoxicity Detection Kit (Roche, Burgess Hill, UK), following the manufacturer's instructions. The mean of three readings for each sample was recorded, with medium only, untreated and dead controls. The readings were normalised for background (minus medium only) and percentage toxicity expressed as [(sample-untreated)/(dead-untreated)×100].

##### RNA Isolation and Reverse Transcription Polymerase Chain Reaction Analysis

As with cell culture, two different techniques were used in the two centres involved in this study for isolating RNA and its analysis by RT-PCR, as described previously [4]. PCR products were analysed on 1.5% (w/v) agarose gels in Tris-borate/EDTA buffer. Skipping efficiencies were determined by quantification of the full length and skipped PCR products by densitometry using GeneTools software (Syngene, Cambridge, UK).

##### Sequence Analysis

RT-PCR products were excised from agarose gels and extracted with a QIAquick gel extraction kit (Qiagen, Crawley, UK). Direct DNA sequencing was carried out by the MRC Genomics Core Facility.

##### Western Blot Analysis of Dystrophin Protein

DMD patient cells, transfected as described and cultured in differentiation medium, were harvested 7, 14 or 21 days post-transfection. 4×10<sup>5</sup> cells were pelleted and resuspended in 50 µl of loading buffer (75 mM Tris-HCl pH 6.8, 15% sodium dodecyl sulphate, 5% β-mercaptoethanol, 2% glycerol, 0.5% bromophenol blue and complete mini protease inhibitor tablet). Samples were incubated at 95° C. for 5 minutes and centrifuged at 18,000×g for 5 minutes. 20 µl of sample was loaded per well in a 6% polyacrylamide gel with 4% stacking gel. Protein from CHQ5B cells differentiated for 7 days was used as a positive control for dystrophin. Gels were electrophoresed for 5 hours at 100V before blotting on nitrocellulose

## US 8,084,601 B2

23

membrane at 200 mA overnight on ice. Blots were stained with Protogold to assess protein loading, then blocked in 10% non-fat milk in PBS with 2% tween (PBST) for 3 hours. Blots were probed with antibodies to dystrophin, NCL-DYS1 (Vector Labs, Peterborough, UK) diluted 1:40 and to dysferlin, Hamlet1 (Vector Labs) diluted 1:300 in 3% non-fat milk/PBST. An anti-mouse, biotinylated secondary antibody (diluted 1:2000; GE Healthcare, Amersham, UK) and streptavidin/horse radish peroxidase conjugated antibody (1:10,000; Dako, Ely, UK) allowed visualisation in a luminol-HRP chemiluminescence reaction (ECL-Plus; GE Healthcare) on Hyperfilm (GE Healthcare), exposed at intervals from 10 seconds to 4 minutes.

## Transgenic Human DMD Mice

A transgenic mouse expressing a complete copy of the human DMD gene has been generated [8, 39]. Experiments were performed at the Leiden University Medical Center, with the authorization of the Animal Experimental Commission (UDEG) of the Medical Faculty of Leiden University as described previously [4].

## Results

Twenty-three PMOs were designed to target exon 53, as described previously [38]. Briefly, SR protein binding motifs, RNA secondary structure and accessibility to binding as determined by hexamer hybridization array analysis, were used as aids to design (FIG. 1). Table 4 summarises the names and target sequence characteristics of these PMOs. These PMOs were initially characterized in normal human skeletal muscle cells (at RHUL). The most active were then directly compared to the PMO targeting the sequence previously identified as most bioactive by Wilton et al. [19] in exon 53-skip-pable patient cells (at UCL), and in the humanised DMD mouse (at LUMC).

## Comparison of PMOs to Exon 53 in Normal Human Skeletal Muscle Cells

An array of seventeen 25mer leashed PMOs were transfected, at a concentration of 500 nM, into normal human skeletal muscle myoblast cultures using lipofectin. Of these seventeen, only four produced consistent levels of exon skipping considered to be above background i.e. over 5% skipping [38], as assessed by densitometric analysis (FIG. 6a). These were PMO-A, -B, -C and -D, which targeted exon 53 at positions +35+59, +38+62, +41+65 and +44+68 respectively. The levels of exon skipping produced were as follows: PMO-A, 12.7%; PMO-B, 9.7%; PMO-C, 10.5%; and PMO-D, 9.0%. When nucleofection was used as a means of introducing naked PMOs into the cells, higher levels of exon skipping were observed for PMO-A and PMO-B only, with 300 nM doses producing 41.2% and 34.3% exon skipping, respectively. The superiority of nucleofection over lipofection has been observed by others (Wells et al., in preparation). However no exon skipping was evident following nucleofection with any of the other naked 25mer PMOs tested (data not shown).

A 3 nt-stepped array of 30mer PMOs was then designed to target the region of exon 53 (position +30 to +74) associated with exon skipping activity by the 25mer PMOs. Following lipofection into normal human skeletal muscle myoblast cultures at a concentration of 500 nM, PMO-G (+30+59), PMO-H (+33+62), PMO-I (+36+65), PMO-J (+39+68) and PMO-K (+42+71) gave reproducible exon skipping above background (FIG. 6b), while PMO-L (+45+74) was inactive. The levels of exon skipping produced were as follows: PMO-G, 37.1%; PMO-H, 44.5%; PMO-I, 27.4%; PMO-J, 33.0%; and PMO-K, 13.0%. The concentration dependence of exon skipping by the more active 30mer PMOs was examined further (FIG. 7a). PMO-H and PMO-I were able to produce

24

convincing skipping at concentrations as low as 25 nM, while PMO-G was active at 50 nM and PMO-J at 75 nM. The exon skipping produced by these 30mer PMOs was shown to be persistent, surviving the lifetime of the cultures (14 days) (FIG. 7b and data not shown). When unleashed 30mer PMOs were introduced into normal muscle cultures by nucleofection, high levels of exon skipping were also observed. For example, at 300 nM, PMO-G and PMO-H gave over 80% skipping of exon 53 (data not shown).

## Comparison of PMOs to Exon 53 in DMD Patient Cells

The PMOs, both 25mer and 30mer, that produced the highest levels of DMD exon 53 skipping in normal skeletal muscle cultures, were then compared to each other for bioactivity in DMD patient (del 45-52) cells, and were also compared to an additional reagent, PMO-M (+39+69), described previously [19]. This comparative evaluation was performed in a blinded fashion. When tested and compared directly at 300 nM doses by nucleofection, PMO-G, PMO-H and PMO-A were most active producing in the order of 60% exon skipping (FIG. 8). The other PMOs tested produced the following exon skipping levels: PMO-I, 45%; PMO-B, 41%; PMO-J, 27%; PMO-M, 26%. All the other PMOs tested gave exon skipping at lower levels of between 10 and 20%.

When the concentration dependence of exon skipping was examined for the most bioactive PMOs, levels approaching 30% were evident for PMO-G and PMO-H at concentrations as low as 25 nM (FIG. 9a, b). Similar levels of skipping were only achieved by PMO-A, PMO-B and PMO-M at 100 nM, while PMO-I needed to be present at 200 nM to produce over 30% exon skipping (FIG. 9a, b). There was no evidence that any of the PMOs tested caused cellular cytotoxicity relative to mock-transfected controls, as assessed by lactate dehydrogenase release into culture medium (results not shown). The exon skipping produced by the six most bioactive PMOs was shown to be persistent, lasting for up to 10 days after transfection, with over 60% exon skipping observed for the lifetime of the cultures for PMO-A, PMO-G and PMO-H (FIG. 10a, b). Exon skipping was shown to persist for 21 days for PMO-A and PMO-G (FIG. 10c).

Western blot analysis of DMD patient (del 45-52) cell lysates, treated in culture with the most bioactive 25mers (PMO-A and PMO-B) and longer PMOs (PMO-G, PMO-H, PMO-I and PMO-M) is shown in FIG. 10e. De novo expression of dystrophin protein was evident with all six PMOs, but was most pronounced with PMO-H, PMO-I, PMO-G and PMO-A, producing 50%, 45%, 33% and 26% dystrophin expression, respectively, relative to the positive control, and seemingly weakest with PMO-B and PMO-M (11% and 17% dystrophin expression respectively, relative to the positive control). However, the limitations of quantifying Western blots of this nature should be taken into account when interpreting the data.

## Comparison of PMOs to Exon 53 in Humanised DMD Mouse

The hDMD mouse is a valuable tool for studying the processing of the human DMD gene in vivo, and as such provides a model for studying the in vivo action of PMOs, prior to clinical testing in patients. PMO-A, PMO-G, PMO-H, PMO-I and PMO-M were injected into the gastrocnemius muscle of hDMD mice, and RNA extracted from the muscles was analysed for exon 53 skipping (FIG. 11). Skipping of exon 53 is evident for each of the PMOs tested; 8% for PMO-A, 7.6% for PMO-I, 7.2% for PMO-G, but to a slightly lower level of 4.8% for PMO-H. PMO-M produced exon skipping levels of less than 1%, which is the detection threshold for the system used.

It should be noted that the levels of exon skipping by each particular PMO was variable. This has been reported previ-

ously [8], and is likely to be due to the poor uptake into the non-dystrophic muscle of the hDMD mouse. However this does not compromise the importance of the finding that the PMOs tested here are able to elicit the targeted skipping of exon 53 in vivo.

Of the 24 PMOs tested, six (PMO-A, PMO-B, PMO-G, PMO-H, PMO-I and PMO-M) produced over 50% targeted skipping of exon 53 either in normal myotubes or in patient myotubes or both. The characteristics of these active PMOs and their target sites are summarised in Table 4. They all showed strong overlap (92%-100%) with the sequence shown to be accessible to binding on the hybridization array analysis, had similar GC content (50%-56%), but varying degrees of overlap (32%-60%) with ESE sites as predicted by Rescue ESE analysis, varying degrees of overlap with ESE sites and ESS sites (60%-86% and 0%-10%, respectively) as predicted by PESX analysis, and all showed overlap with two SR binding motifs (SF2/ASF, as defined by the BRCA1 algorithm, and SRp40). It should be noted that PMO-J, -K, -L and M had a common SNP of exon 53 (c7728C>T) in the last, fourth to last, seventh to last and second to last base, respectively of their target sites. There is the potential that this allelic mismatch could influence the binding and bioactivity of these PMOs. However, the more active PMOs (-A, -B, -G, -H and -I) all had their target sites away from the SNP, and the possible effect of a mismatch weakening binding and bioactivity is removed, and allows definitive comparisons between these PMOs to be made.

#### Discussion

The putative use of AOs to skip the exons which flank out-of-frame deletions is fast becoming a reality in the experimental intervention of DMD boys. Indeed the restoration of dystrophin expression in the TA muscle of four patients, injected with a 2'OMePS AO optimised to target exon 51 of the DMD gene, has been reported recently [11]. Moreover a clinical trial using a PMO targeting exon 51 has recently been completed in seven DMD boys in the UK (Muntoni et al, in preparation). However, the targeted skipping of exon 51 would have the potential to treat only 13% of DMD patients with genomic deletions on the Leiden database [37]. There is therefore a definite requirement for the optimisation of AOs to target other exons commonly mutated in DMD.

Although there have been many large screens of AO bioactivity in vitro [18, 19, 38, 40], no definite rules to guide AO design have become apparent. Previous studies in the mdx mouse model of DMD showed that AOs that targeted the donor splice site of exon 23 of the mouse DMD gene restored dystrophin expression [7]. However the targeting of AOs to the donor splice sites of exon 51 of the human DMD gene was ineffective at producing skipping [4], and it has been suggested that the 'skippability' of human DMD exons has no correlation with the predicted strength of the donor splice site [41]. It has been reported that exon skipping could be induced by the targeting of AOs to exonic splicing enhancer (ESE) motifs [18, 40]. These motifs are recognised by SR proteins, which facilitate exon splicing by recruiting splicing effectors (U1 and U2AF) to the donor splice site (reviewed by Cartegni et al.) [42]. However these motifs are divergent, poorly defined, their identification complex, and their strength as AO design tools dubious [38].

A comparative study of 66 PMOs designed to five different DMD exons demonstrated the significance of RNA secondary structure in relation to accessibility of the PMO target site and subsequent PMO bioactivity [38], as assessed by mfold software prediction of secondary structure [25], and a hybrid-

ization screen against a hexamer array [38]. PMOs that bound to their target more strongly, either as a result of being longer or in being able to access their target site more directly, were significantly more bioactive. The influence of AO length on bioactivity has been reported elsewhere [4, 30], and is further confirmed in the present study; all 30mers tested were more bioactive relative to their 25mer counterpart. The fact that 30mer PMOs were more bioactive than 25mer PMOs targeted to the same open/accessible sites on the exon, would suggest that strength of binding of PMO to the target site may be the most important factor in determining PMO bioactivity. These thermodynamic considerations have also been reported in a complementary study of 2'OMePS AOs [40]. However, it has also been reported that two overlapping 30mers were not as efficient as a 25mer at skipping mouse exon 23, indicating that oligomer length may only be important in some cases [4].

To ensure that the analysis of PMOs for the targeted skipping of exon 53 was not biased by any particular design strategy, seventeen 25mer PMOs were designed to cover the whole of exon 53, with stepwise arrays over suggested bioactive target sites, and then subsequently six 30mer PMOs were designed to target the sequence of exon 53 that showed an association with exon skipping for the 25mers tested. PMOs were designed and tested independently by two different groups (at RHUL and UWA), and then efficacy of the best thirteen sequences confirmed by two other independent groups (at UCL and LUMC). Such a collaborative approach has been used previously as a way of validating target sequences in DMD [4]. Human myoblasts allowed the controlled in vitro comparison of PMO sequences, and confirmation of skipping of exon 53 at the RNA level by certain PMOs in both normal cells and, perhaps more importantly, in DMD patient cells with a relevant mutation. These results were further borne out by the expression of dystrophin protein in the DMD cells treated with specific PMOs. Use of the humanised DMD mouse provided an in vivo setting to confirm correct exon exclusion prior to any planned clinical trial. The combined use of these three different systems (normal cells, patient cells and hDMD mouse) as tests of PMO bioactivity provided a reliable and coherent determination of optimal sequence(s) for the targeted skipping of exon 53.

When considering the data presented here as a whole, the superiority of the PMO targeting the sequence +30+59 (PMO-G, or h53A30/1), is strongly indicated. In normal myoblasts, nucleofection of PMO-G (300 nM) and liposomal-carrier mediated transfection of leashed PMO-G (500 nM) produced over 80% and over 50% skipping of exon 53, respectively, implying that it acts extremely efficiently within the cell. This was confirmed in patient cells. Indeed, this PMO generates the highest levels of exon skipping in patient cells over a range of concentrations (up to 200 nM) and, most important therapeutically, exerts its activity at concentrations as low as 25 nM. The exon skipping activity of this PMO is also persistent, with over 70% exon skipping for 7 days in culture, and over 60% exon skipping for up to three weeks. This would have important safety and cost implications as a genetic therapy for DMD patients with the appropriate deletions. PMO-G was also shown to skip exon 53 correctly in vivo. These RNA results were further confirmed by the detection of dystrophin protein at a high level in protein extracts from patient cells treated with PMO-G. Previous studies by the Leiden group [18] suggest that the optimal 2'OMePS AO is targeted to the sequence +46+63 of exon 53, producing exon skipping in up to 25% of transcripts in cultured cells and 7% in the hDMD mouse. This 2'OMePS AO shows some

## US 8,084,601 B2

27

degree of overlap with the optimal PMOs reported here which strengthens our findings. The reason that our optimal PMO is more specific could be a (combined) consequence of the different AO chemistries, length of AO used, and the absolute target site of AO.

28

The sequence h53A30/1 we have identified appears to be more efficient than any of the previously reported AOs designed to skip exon 53 of the DMD gene, and this PMO therefore represents, at the present time, the optimal sequence for clinical trials in DMD boys.

TABLE 4

Table 4: Table summarizing the characteristics of PMOs used										
PMO	Position			Exon- PMO binding	PMO- PMO binding	%	Ends in open	% overlap with hybrid.	# Rescue ESE	% overlap with Rescue
	Start	End	% GC	energy	energy	open <sup>b</sup>	loops <sup>b</sup>	peak	sites	ESE
A h53A1	+35	+59	52	-38.6	-17.4	50	2	92	7	56
B h53A2	+38	+62	56	-36.1	-17.4	46.7	1	100	4	32
C h53A3	+41	+65	56	-36.7	-13.7	36.7	0	0	3	32
D h53A4	+44	+68	48	-34.3	-8.5	20	0	100	4	28
E h53A5	+47	+71	48	-35.5	-8.5	43.3	2	100	3	36
F h53A6	+50	+74	48	-35.3	-8.5	43.3	2	92	2	36
N h53B1	+69	+93	28	-22.1	-12.1	53.3	1	0	5	56
O h53B2	+80	+104	48	-30.1	-11.3	23.3	1	0	5	60
P h53B3	+90	+114	48	-34.5	-5.5	48	2	0	8	72
Q h53C1	+109	+133	48	-32.4	-9.8	46.7	2	0	6	52
R h53C2	+116	+140	56	-31.3	-12.7	33.3	1	0	1	24
S h53C3	+128	+152	60	-34.6	-13.7	26.7	1	0	1	24
T h53D1	+149	+173	52	-34.1	-13.4	30	1	0	4	40
U h53D2	+158	+182	48	-36.5	-14.5	40	2	0	6	44
V h53D3	+170	+194	36	-34.3	-11.2	40	1	0	9	64
W h53D4	+182	+206	32	-30.9	-9.2	63.3	1	0	16	96
X h53D5	+188	+212	36	-31.5	-3.3	66.7	1	0	14	92
G h53A30/1	+30	+59	50	-48.1	-17.4	56.7	1	92	9	60
H h53A30/2	+33	+62	53	-45.1	-17.4	63.3	1	100	8	53
I h53A30/3	+36	+65	53	-44.6	-17.4	53.3	1	100	6	43
J h53A30/4	+39	+68	50	-43.4	-17.4	43.3	1	100	4	43
K h53A30/5	+42	+71	47	-42.4	-11.3	46.7	1	100	5	47
L h53A30/6	+45	+74	47	-42.3	-8.5	56.7	1	100	5	48
M H53A	+39	+69	52	-48.5	-17.4	48.4	2	100	4	45

PMO	% overlap with		ESE finder values over threshold <sup>c</sup>						
	PESE	PESS	SF2/ASF	BRCA1	SC35	SRp40	SRp55	Tra2B	9G8
A h53A1	84	0	6.58	7.26	0	3.12	0	24.04	19.02
B h53A2	72	0	6.58	7.26	0	3.12	0	7.25	19.02
C h53A3	60	0	6.58	7.26	0	3.12	0	7.25	11.9
D h53A4	48	8	6.58	7.26	0	3.12	0	7.25	11.9
E h53A5	36	20	6.58	7.26	0	3.12	0	7.25	11.9
F h53A6	28	32	6.58	7.26	0	0	0	7.25	11.9
N h53B1	40	40	0	9.26	3.62	10.66	0	5.06	1.1
O h53B2	60	0	0	9.26	3.62	4.73	0	5.06	8.28
P h53B3	64	0	3.49	9.26	3.44	4.73	0	24.04	28.68
Q h53C1	72	0	4.19	6.72	0	2.04	0	24.04	28.68
R h53C2	60	0	4.19	6.72	10.2	4.38	0	0	8.28
S h53C3	32	0	3.49	6.41	10.2	4.38	6.86	0	14.18
T h53D1	32	0	0.52	0	18.68	0	6.86	0	12.71
U h53D2	32	0	0.52	1.8	18.68	0.42	0	0	12.71
V h53D3	0	0	0	1.8	0	6.95	0	24.04	10.49
W h53D4	24	0	8.5	11.95	0	7.67	0.33	24.04	7.14
X h53D5	44	0	8.5	11.95	0	7.67	0.33	24.04	7.14
G h53A30/1	86	0	6.58	7.26	0	3.12	0	24.04	19.02
H h53A30/2	77	0	6.58	7.26	0	3.12	0	24.04	19.02
I h53A30/3	67	0	6.58	7.26	0	3.12	0	24.04	19.02
J h53A30/4	57	7	6.58	7.26	0	3.12	0	7.25	11.9
K h53A30/5	47	17	6.58	7.26	0	3.12	0	7.25	11.9
L h53A30/6	37	27	6.58	7.26	0	3.12	0	7.25	11.9
M H53A	58	10	6.58	7.26	0	3.12	0	7.25	11.9

Characteristics of the PMOs and their target sites listed.

<sup>b</sup>calculated as % of PMO target site in open structures on predicted RNA secondary structure obtained using MFOLD analysis. The position of the PMO target sites relative to open loops in the RNA secondary structure is listed (0 = no ends in open loops, 1 = one end in an open loop, 2 = both ends in open loops).

<sup>c</sup>In the analyses, SR binding sites were predicted using splice sequence finder (<http://www.umd.be/SSF/>) software. Values above threshold are given for PMOs whose target sites cover 50% or more of potential binding sites for SF2/ASF, BRCA1, SC35, SRp40, SRp55, Tra2B and 9G8

## US 8,084,601 B2

29

## REFERENCES

1. Hoffmann E P, Brown R H, Kunkel L M (1987) Dystrophin: The protein product of the Duchenne muscular dystrophy locus. *Cell*; 51: 919-928.
2. Den Dunnen J T, Grootsscholten P M, Bakker E, Blonden L A, Ginjaar H B, Wapenaar M C, et al. (1989). Topography of the Duchenne muscular dystrophy (DMD) gene: FIGE and cDNA analysis of 194 cases reveals 115 deletions and 13 duplications. *Am J Hum Genet*; 45: 835-847.
3. van Deutekom J C, Bremmer-Bout M, Janson A A, Ginjaar I B, Baas F, den Dunnen J T, et al. (2001). Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells. *Hum Mol Genet*; 10: 1547-1554.
4. Arechavala-Gomez V, Graham I R, Popplewell L J, Adams A M, Aartsma-Rus A, Kinali M, et al. (2007). Comparative analysis of antisense oligonucleotide sequences for targeted skipping of exon 51 during pre-mRNA splicing in human muscle. *Hum Gene Ther*; 18: 798-810.
5. Mann C J, Honeyman K, Cheng A J, Ly T, Lloyd F, Fletcher S, et al. (2001). Antisense-induced exon skipping and synthesis of dystrophin in the mdx mouse. *Proc Natl Acad Sci USA*; 98: 42-47.
6. Lu Q L, Mann C J, Lou F, Bou-Gharios G, Morris G E, Xue S A, et al. (2003). Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse. *Nat Med*; 9: 1009-1014.
7. Graham I R, Hill V J, Manoharan M, Inamati G B, Dickson G (2004). Towards a therapeutic inhibition of dystrophin exon 23 splicing in mdx mouse muscle induced by antisense oligonucleotides (splicomers): target sequence optimisation using oligonucleotide arrays. *J Gene Med*; 6: 1149-1158.
8. Bremmer-Bout M, Aartsma-Rus A, de Meijer E J, Kaman W E, Janson A A, Vossen R H, et al. (2004). Targeted exon skipping in transgenic hDMD mice: A model for direct preclinical screening of human-specific antisense oligonucleotides. *Mol Ther*; 10: 232-240.
9. Jearawiriyapaisarn N, Moulton H M, Buckley B, Roberts J, Sazani P, Fucharoen S, et al. (2008). Sustained dystrophin expression induced by peptide-conjugated morpholino oligomers in the muscles of mdx mice. *Mol. Ther.* June 10 (Epub).
10. Bertoni C. (2008). Clinical approaches in the treatment of Duchenne muscular dystrophy (DMD) using oligonucleotides. *Front Biosci*; 13: 517-527.
11. van Deutekom J C, Janson A A, Ginjaar I B, Franzhuzen W S, Aartsma-Rus A, Bremmer-Bout M, et al. (2007). Local antisense dystrophin restoration with antisense oligonucleotide PRO051. *N Eng J Med*; 357: 2677-2687.
12. Gebbski B L, Mann C J, Fletcher S, Wilton S D (2003). Morpholino antisense oligonucleotide induced dystrophin exon 23 skipping in mdx mouse muscle. *Hum Mol Genet*; 12: 1801-1811.
13. Alter J, Lou F, Rabinowitz A, Yin H, Rosenfeld J, Wilton S D, et al. (2006). Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. *Nat Med*; 12: 175-177.
14. Fletcher S, Honeyman K, Fall A M, Harding P L, Johnsen R D, Wilton S D (2006). Dystrophin expression in the mdx mouse after localized and systemic administration of a morpholino antisense oligonucleotide. *J Gene Med*; 8: 207-216.
15. McClorey G, Fall A M, Moulton H M, Iversen P L, Rasko J E, Ryan M, et al. (2006). Induced dystrophin exon skipping in human muscle explants. *Neuromus Disorders*; 16: 583-590.
16. McClorey G, Moulton H M, Iversen P L, Fletcher S, Wilton S D (2006). Antisense oligonucleotide-induced exon skipping restores dystrophin expression in vitro in a canine model of DMD. *Gene Ther*; 13:1373-1381.
17. Arora V, Devi G R, Iversen P L (2004). Neutrally charged phosphorodiamidate morpholino antisense oligomers: uptake, efficacy and pharmacokinetics. *Curr Pharm Biotechnol*; 5: 431-439.
18. Aartsma-Rus A, De Winter C L, Janson A A M, Kaman W E, van Ommen G-J B, Den Dunnen J T, et al. (2005). Functional analysis of 114 exon-internal AONs for targeted DMD exon skipping: Indication for steric hindrance of SR protein binding sites. *Oligonucleotides*; 15: 284-297.
19. Wilton S D, Fall A M, Harding P L, McClorey G, Coleman C, Fletcher S (2007). Antisense oligonucleotide-induced exon skipping across the human dystrophin gene transcript. *Mol Ther*; 15: 1288-1296.
20. Cartegni L, Wang J, Zhu Z, Zhang M Q, Krainer A R (2003). ESEfinder: A web resource to identify exonic splicing enhancers. *Nucleic Acids Res*; 31: 3568-3571.
21. Smith P J, Zhang C, Wang J, Chew S L, Zhang M O, Krainer A R (2006). An increased specificity score matrix for the prediction of SF2/ASF-specific exonic splicing enhancers. *Human Mol Genet*; 15: 2490-2508.
22. Zhang X H, Chasin L H (2004). Computational definition of sequence motifs governing constitutive exon splicing. *Genes Dev*; 18: 1241-1250.
23. Zhang X H, Leslie C S, Chasin L A (2005). Computational searches for splicing signals. *Methods*; 37: 292-305.
24. Fairbrother W G, Yeh R F, Sharp P A, Burge C B (2002). Predictive identification of exonic splicing enhancers in human genes. *Science*; 297: 1007-1013.
25. Mathews D H, Sabina J, Zuker M, Turner D H (1999). Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J Mol Biol*; 288: 911-940.
26. Aartsma-Rus A, Bremmer-Bout M, Janson A A M, den Dunnen J T, van Ommen G-J B, van Deutekom J C T (2002). Targeted exon skipping as a potential gene correction therapy for Duchenne muscular dystrophy. *Neuromus Disorders*; 12: 871-877.
27. Aartsma-Rus A, Kaman W E, Weij R, den Dunnen J T, van Ommen G J, van Deutekom J C. (2006). Exploring the frontiers of therapeutic exon skipping for Duchenne muscular dystrophy by double targeting within one or multiple exons. *Mol Ther*; 14: 401-407.
28. Adams A M, Harding P L, Iversen P L, Coleman C, Fletcher S, Wilton S D. (2007). Antisense oligonucleotide induced exon skipping and the dystrophin gene transcript: cocktails and chemistries. *BMC Mol Biol*; 8: 57.
29. Vickers T A, Wyatt J R, Freier S M (2000). Effects of RNA secondary structure on cellular antisense activity. *Nucleic Acids Res*; 28: 1340-1347.
30. Harding P L, Fall A M, Honeyman K, Fletcher S, Wilton S D (2007). The influence of antisense oligonucleotide length on dystrophin exon skipping. *Mol Ther*; 15: 157-166.
31. Wee K B, Pramono Z A D, Wang J L, MacDorman K F, Lai P S, Yee W C (2008). Dynamics of co-translational pre-mRNA folding influences the induction of dystrophin exon skipping by antisense oligonucleotides. *Plos one*; 3: e1844.

30

## US 8,084,601 B2

## 31

32. Fairbrother W G, Yeo G W, Yeh R, Goldstein P, Mawson M, Sharp P A, et al. (2004). RESCUE-ESE identifies candidate exonic splicing enhancers in vertebrate exons. *Nucleic Acids Res*; 32: W187-190.
33. Patzel V, Steidl R, Kronenwell R, Haas R, Sczakiel G (1999). A theoretical approach to select effective antisense oligodeoxyribonucleotides at high statistical probability. *Nucleic Acids Res*; 27: 4328-4334.
34. Ihaka R, Gentleman R C (1996). R: A Language for Data Analysis and Graphics. *Journal of Computational and Graphical Statistics*; 15: 999-1013.
35. Moulton H M, Fletcher S, Neuman B W, McClorey G, Stein D A, Abes S, Wilton S D, Buchmeier M J, Lebleu B, Iversen P L (2007). Cell-penetrating peptide-morpholino conjugates alter pre-mRNA splicing of DMD (Duchenne muscular dystrophy) and inhibit murine coronavirus replication in vivo. *Biochem. Soc. Trans.* 35: 826-8.
36. Jearawiriyapaisarn N, Moulton H M, Buckley B, Roberts J, Sazani P, Fucharoen S, Iversen P L, Kole R (2008). Sustained Dystrophin Expression Induced by Peptide-conjugated Morpholino Oligomers in the Muscles of mdx Mice. *Mol. Ther.* June 10. Epub ahead of print.

## 32

37. Aartsma-Rus A, Fokkema I, Verschuuren J, Ginjaar I, van Deutekom J, van Ommen G J et al. Theoretic applicability of antisense-mediated exon skipping for Duchenne muscular dystrophy mutations. *Hum Mutation* 2009; Jan. 20 (Epub).
38. Popplewell L J, Trollet C, Dickson G, Graham I R. Design of phosphorodiamidate morpholino oligomers (PMOs) for the induction of exon skipping of the human DMD gene. *Mol Ther* 2009; Jan. 13 (Epub).
39. 'tHoen PAC, de Meijer E J, Boer J M, Vossen R H, Turk R, Maatman R G et al. (2008) Generation and characterization of transgenic mice with the full-length human DMD gene. *J Biol Chem*; 283: 5899-5907.
40. Aartsma-Rus A, van Vliet L, Hirschi M, Janson A A, Heemskerk H, de Winter C L, et al. Guidelines for antisense oligonucleotide design and insight into splice-modulating mechanisms. *Mol Ther* 2008; Sep. 23 (Epub).
41. Aartsma-Rus A, van Ommen G J. Antisense-mediated exon skipping: A versatile tool with therapeutic and research applications. *RNA* 2007; 13: 1-16.
42. Cartegni L, Chew S L, Krainer A R. Listening to silence and understanding nonsense: Exonic mutations that affect splicing. *Nat Rev Genet.* 2002; 3: 285-298.

## SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 26

<210> SEQ ID NO 1  
 <211> LENGTH: 30  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
 analogs thereof, especially with same base  
 sequence  
 <221> NAME/KEY: misc\_feature  
 <222> LOCATION: (1)...(30)  
 <223> OTHER INFORMATION: n = T or U

<400> SEQUENCE: 1

ngaaaacgcc gccannncnc aacagancng 30

<210> SEQ ID NO 2  
 <211> LENGTH: 30  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
 analogs thereof, especially with same base  
 sequence  
 <221> NAME/KEY: misc\_feature  
 <222> LOCATION: (1)...(30)  
 <223> OTHER INFORMATION: n = T or U

<400> SEQUENCE: 2

canaangaaa acgcccgcann nncncaacag 30

<210> SEQ ID NO 3  
 <211> LENGTH: 30  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
 analogs thereof, especially with same base  
 sequence  
 <221> NAME/KEY: misc\_feature  
 <222> LOCATION: (1)...(30)  
 <223> OTHER INFORMATION: n = T or U

US 8,084,601 B2

33

34

-continued

---

<400> SEQUENCE: 3

ngnncagcnn cngnagcca cngannaaan

30

<210> SEQ ID NO 4

<211> LENGTH: 30

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
analogs thereof, especially with same base  
sequence

<221> NAME/KEY: misc\_feature

<222> LOCATION: (1)...(30)

<223> OTHER INFORMATION: n = T or U

<400> SEQUENCE: 4

cagnnngccg cngcccaang ccancngga

30

<210> SEQ ID NO 5

<211> LENGTH: 30

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
analogs thereof, especially with same base  
sequence

<221> NAME/KEY: misc\_feature

<222> LOCATION: (1)...(30)

<223> OTHER INFORMATION: n = T or U

<400> SEQUENCE: 5

nngccgcngc ccaangccan cnggagnnc

30

<210> SEQ ID NO 6

<211> LENGTH: 30

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
analogs thereof, especially with same base  
sequence

<221> NAME/KEY: misc\_feature

<222> LOCATION: (1)...(30)

<223> OTHER INFORMATION: n = T or U

<400> SEQUENCE: 6

ngcngcncnn nnccagggnnc aagngggana

30

<210> SEQ ID NO 7

<211> LENGTH: 30

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
analogs thereof, especially with same base  
sequence

<221> NAME/KEY: misc\_feature

<222> LOCATION: (1)...(30)

<223> OTHER INFORMATION: n = T or U

<400> SEQUENCE: 7

cnnnnagnng cngcncnnnn ccaggnncaa

30

<210> SEQ ID NO 8

<211> LENGTH: 30

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

US 8,084,601 B2

35

36

-continued

---

<220> FEATURE:  
<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
analogs thereof, especially with same base  
sequence  
<221> NAME/KEY: misc\_feature  
<222> LOCATION: (1)...(30)  
<223> OTHER INFORMATION: n = T or U  
  
<400> SEQUENCE: 8  
  
cnnnnncnnnn agnngcngcn cnnnnccagg 30  
  
<210> SEQ ID NO 9  
<211> LENGTH: 30  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
analogs thereof, especially with same base  
sequence  
<221> NAME/KEY: misc\_feature  
<222> LOCATION: (1)...(30)  
<223> OTHER INFORMATION: n = T or U  
  
<400> SEQUENCE: 9  
  
nnagnngcng cncnnnncca ggnncaagn 30  
  
<210> SEQ ID NO 10  
<211> LENGTH: 30  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
analogs thereof, especially with same base  
sequence  
<221> NAME/KEY: misc\_feature  
<222> LOCATION: (1)...(30)  
<223> OTHER INFORMATION: n = T or U  
  
<400> SEQUENCE: 10  
  
cngnngccnc cggnnncngaa gngnncnng 30  
  
<210> SEQ ID NO 11  
<211> LENGTH: 30  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
analogs thereof, especially with same base  
sequence  
<221> NAME/KEY: misc\_feature  
<222> LOCATION: (1)...(30)  
<223> OTHER INFORMATION: n = T or U  
  
<400> SEQUENCE: 11  
  
caacngnngc cnccggnncn gaaggngnnc 30  
  
<210> SEQ ID NO 12  
<211> LENGTH: 30  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
analogs thereof, especially with same base  
sequence  
<221> NAME/KEY: misc\_feature  
<222> LOCATION: (1)...(30)  
<223> OTHER INFORMATION: n = T or U  
  
<400> SEQUENCE: 12  
  
nngccnccgg nncngaaggn gnnchnngnac 30

US 8,084,601 B2

37

38

-continued

---

<210> SEQ ID NO 13  
<211> LENGTH: 30  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
analogs thereof, especially with same base  
sequence  
  
<400> SEQUENCE: 13  
  
tgaaaaacgcc gccatttctc aacagatctg 30  
  
<210> SEQ ID NO 14  
<211> LENGTH: 30  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
analogs thereof, especially with same base  
sequence  
  
<400> SEQUENCE: 14  
  
cataatgaaa acgccgccat ttctcaacag 30  
  
<210> SEQ ID NO 15  
<211> LENGTH: 30  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
analogs thereof, especially with same base  
sequence  
  
<400> SEQUENCE: 15  
  
tgttcagctt ctgtagcca ctgattaaat 30  
  
<210> SEQ ID NO 16  
<211> LENGTH: 30  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
analogs thereof, especially with same base  
sequence  
  
<400> SEQUENCE: 16  
  
cagtttgccg ctgccaatg ccatcctgga 30  
  
<210> SEQ ID NO 17  
<211> LENGTH: 30  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
analogs thereof, especially with same base  
sequence  
  
<400> SEQUENCE: 17  
  
ttgccgctgc ccaatgccat cctggagttc 30  
  
<210> SEQ ID NO 18  
<211> LENGTH: 30  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
analogs thereof, especially with same base  
sequence

US 8,084,601 B2

39

40

-continued

<400> SEQUENCE: 18

tgctgctctt ttccagggtc aagtgggata

30

<210> SEQ ID NO 19

<211> LENGTH: 30

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
analogs thereof, especially with same base  
sequence

<400> SEQUENCE: 19

cttttagttg ctgctctttt ccaggttcaa

30

<210> SEQ ID NO 20

<211> LENGTH: 30

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
analogs thereof, especially with same base  
sequence

<400> SEQUENCE: 20

cttttctttt agttgctgct cttttccagg

30

<210> SEQ ID NO 21

<211> LENGTH: 30

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
analogs thereof, especially with same base  
sequence

<400> SEQUENCE: 21

ttagttgctg ctcttttcca ggttcaagtg

30

<210> SEQ ID NO 22

<211> LENGTH: 30

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
analogs thereof, especially with same base  
sequence

<400> SEQUENCE: 22

ctgttgcttc cggttctgaa ggtgttcttg

30

<210> SEQ ID NO 23

<211> LENGTH: 30

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or  
analogs thereof, especially with same base  
sequence

<400> SEQUENCE: 23

caactgttgc ctccggttct gaaggtgttc

30

<210> SEQ ID NO 24

<211> LENGTH: 30

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

US 8,084,601 B2

41

42

-continued

---

```

<220> FEATURE:
<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or
analogs thereof, especially with same base
sequence

<400> SEQUENCE: 24

ttgectccgg ttctgaaggt gttctgttac                               30

<210> SEQ ID NO 25
<211> LENGTH: 210
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or
analogs thereof, especially with same base
sequence

<400> SEQUENCE: 25

tgaagaatt cagaatcagt gggatgaagt acaagaacac cttcagaacc ggaggcaaca      60
gttgaatgaa atgttaaagg attcaacaca atggctggaa gctaaggaag aagctgagca    120
ggtcttagga caggccagag ccaagcttga gtcattggaag gagggtcctt atacagtaga    180
tgcaatccaa aagaaaatca cagaaaccaa                                     210

<210> SEQ ID NO 26
<211> LENGTH: 311
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: oligonucleotides or oligonribonucleotides or
analogs thereof, especially with same base
sequence

<400> SEQUENCE: 26

cctccagact agcatttact actatatatt ttttttctt tttattctag ttgaaagaat      60
tcagaatcag tgggatgaag tacaagaaca ccttcagaac cggaggcaac agttgaatga    120
aatgttaaag gattcaacac aatggctgga agctaaggaa gaagctgagc aggtcttagg    180
acaggccaga gccaaagcttg agtcatggaa ggagggtccc tatacagtag atgcaatcca    240
aaagaaaatc acagaaacca aggttagtat caaagaacct ttttaaaata aaatactggt    300
tacatttgat a                                                         311

```

---

The invention claimed is:

1. An oligomer for ameliorating DMD, the oligomer comprising at least 25 contiguous bases of a base sequence selected from the group consisting of:

a)

(SEQ ID NO: 10)  
CXG XXG CCX CCG GXX CXG AAG GXG XXC XXG;

b) CAA CXG XXG CCX CCG GXX CXG AAG GXG XXC;  
and

c) XXG CCX CCG GXX CXG AAG GXG XXC XXG XAC,

wherein X=U or T, wherein the oligomer's base sequence can vary from the above sequence at up to two base positions, and wherein the molecule can bind to a target site to cause exon skipping in an exon of the dystrophin gene.

2. The oligomer according to claim 1, wherein the exon of the dystrophin gene at which exon skipping is caused is exon 53.

3. The oligomer according to claim 1, wherein the oligomer causes an exon skipping rate of at least 50%.

4. The oligomer according to claim 1, wherein the oligomer is between 25 and 35 bases in length.

5. The oligomer according to claim 1, wherein the oligomer is 30 bases in length.

6. The oligomer according to claim 1, wherein the oligomer is conjugated to or complexed with a distinct chemical entity.

7. The oligomer according to claim 1, wherein the oligomer is a phosphorodiamidate morpholino oligonucleotide (PMO).

8. A vector for ameliorating DMD, the vector encoding an oligomer according to claim 1, wherein when introduced into a human cell the oligomer is expressed.

9. A pharmaceutical composition for ameliorating DMD, the composition comprising an oligomer according to claim 1

US 8,084,601 B2

**43**

or a vector according to claim **8**, and a pharmaceutically acceptable carrier, adjuvant or vehicle.

**10.** A pharmaceutical composition according to claim **9** comprising a plurality of oligomers or vectors encoding oli-

**44**

gomers, or a combination of the oligomers and vectors, wherein the oligomers and/or vectors in the pharmaceutical composition cause skipping in a plurality of exons.

\* \* \* \* \*

# Exhibit 39



		Search
Home		
Products and Applications		
Ordering		
Customer Support		
Morpholino Publications Database		
FAQ		
About Us		
Contact Us		

- [Properties](#)
  - [Solubility](#)
  - [Stability](#)
  - [Binding Affinity](#)
  - [Efficacy: Cell-Free](#)
  - [Efficacy: In Cells](#)
- [Specificity](#)
- [Commercial Source](#)
- [References](#)

## [Table of Contents](#)

## General Information

**GENE TOOLS, LLC** is the exclusive commercial source for research quantities of Morpholino oligos.

In Japan, Morpholino oligos are distributed solely by [Funakoshi Co.](#)

Morpholino oligos were devised by James Summerton in 1985 and were developed at ANTIVIRALS Inc. (now [Sarepta Therapeutics Inc.](#)), the pioneer antisense company founded by Summerton in 1980. James

Summerton, Ph.D. is now the Manager of **GENE TOOLS, LLC**.

Morpholinos and the subunits used in their assembly have been awarded multiple pending and issued US and international patents, including particularly US Patents 5,142,047 and 5,185,444.

[Table of Contents](#)

## Design

By 1984 a growing concern in the then-emerging antisense field was that antisense therapeutics might never be commercially viable because of their very high production costs. Two key factors in the high cost of DNA analogs are: 1) the limited availability and high cost of their deoxyribonucleoside precursors; and, 2) the complexity and expense associated with coupling to hydroxyls, required in forming the phosphoester intersubunit linkages of most nucleic acid analogs.

In 1985 Summerton devised the Morpholino structural type to circumvent both of these cost problems [Summerton, 1989; Summerton, 1990]. This is achieved by starting with much less expensive ribonucleosides and introducing an amine via a relatively simple ribose-to-morpholine transformation. The resulting Morpholino subunits can be assembled into antisense oligos via simple and efficient coupling to the morpholine nitrogen, without the expensive catalysts and post-coupling oxidation steps required in the production of most nucleic acid analogs.

Early molecular modeling suggested that Morpholinos should effectively bind complementary nucleic acids. This predicted good binding affinity has been confirmed, as described later herein. Additionally, their unnatural structures and lack of anionic sites on the backbone were expected to preclude degradation by nucleases. This also has been confirmed by extensive experiments noted in a subsequent section.

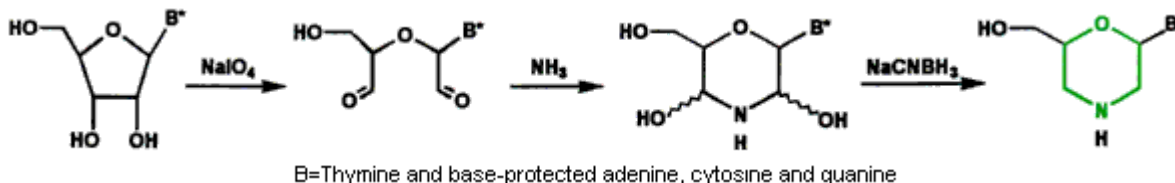
[Table of Contents](#)

## Preparation

### 1. Subunit Synthesis

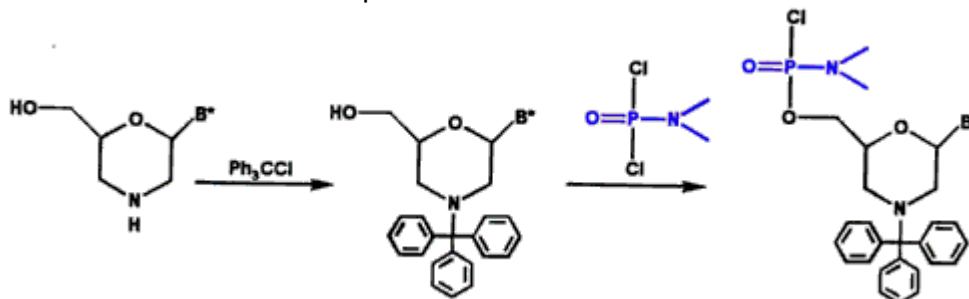
The first step in preparing Morpholino oligos is to convert ribonucleosides to Morpholino subunits as shown in Figure 1.

Figure 1. Ribonucleoside to Morpholino Transformation



Morpholino subunits are subsequently protected with a trityl and activated with our phosphoroamidate linking agent, as shown in Figure 2.

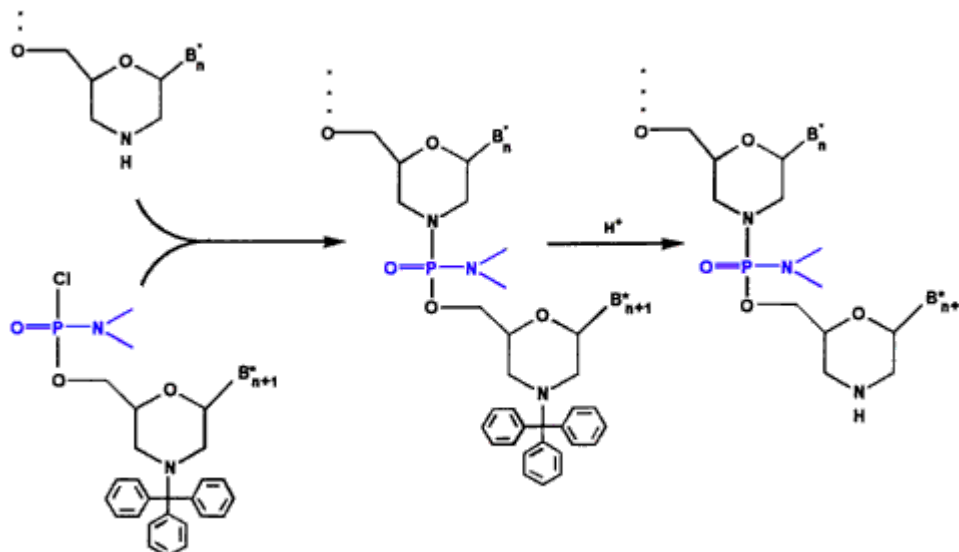
Figure 2. Protection and Activation of Morpholino Subunit



## 2. Oligo Assembly

As shown in Figure 3, we assemble the activated subunits into oligos of defined sequence on a solid-phase support using a simple two-reaction cycle, with intervening washes. The first reaction entails adding activated subunit. The second reaction entails detritylation to prepare the chain terminus for the next subunit addition.

Figure 3. Oligo Assembly Cycle



## 3. Oligo Processing

Concentrated  $\text{NH}_4\text{OH}$  is used to cleave the oligo from the resin and remove protective groups from the nucleobases. The finished oligo is cleaned up by filtration and selective precipitation. The oligo is resuspended in pure water. An aliquot is taken for MALDI-TOF mass spectrometry and another aliquot is quantitated spectrophotometrically at 265 nm in 0.1 N HCl. UV spectra are taken at acidic pH to preclude errors due to hypochromicity resulting from stacking of the bases.

The value from this UV spectral quantitation is used to calculate oligo concentration, after which 300 nanoMoles of oligo is placed into a vial and freeze dried. The vial is sealed and baked to give a sterile freeze-dried oligo.

To use that oligo, one simply adds a defined volume of sterile water to form a sterile pre-quantitated stock solution ready to use in antisense experiments.

[Table of Contents](#)

# Properties

## 1. Solubility

An antisense oligo should have good water solubility to assure good access to its target sequence within the cell. Conventional wisdom in the antisense field is that non-ionic antisense oligos (such as Morpholinos) invariably show poor water solubility. Contrary to that conventional wisdom, it has been found that if the nucleobases in a non-ionic antisense oligo stack poorly (determined by circular dichroism studies) then that oligo has poor water solubility, but if the nucleobases are well stacked in aqueous solution then that oligo can show excellent water solubility.

This stacking/solubility correlation is evidenced by the poor stacking of carbamate-linked DNA analogs and carbamate-linked Morpholino oligos [Kang et al., 1992] and their corresponding poor aqueous solubilities [Stirchak, Summerton & Weller, 1987; Stirchak, Summerton & Weller, 1989]. In contrast, the

standard non-ionic phosphorodiamidate-linked Morpholino oligos shown in Figure 1 exhibit excellent base stacking - even better than that in DNA [Kang et al., 1992] and they have very good water solubility - evidenced by dissolution of 263 mg of a representative 22-mer in 1 ml of water without reaching saturation [Summerton & Weller, 1997a].

It is postulated that the low water solubility of non-ionic oligos with poorly-stacked bases is a consequence of the difficulty of inserting the hydrophobic faces of the unstacked bases into an aqueous environment. Conversely, the high water solubility of non-ionic phosphorodiamidate-linked Morpholinos is likely due to effective shielding of those hydrophobic faces from the polar solvent because of the exceptionally good stacking of the bases.

## 2. Stability

For optimal activity an antisense oligo should be completely resistant to nucleases. DNA antisense oligos are degraded in serum and within cells in a matter of minutes.

Currently-popular thiophosphates (S-DNAs) are appreciably more stable than DNA. Nonetheless, S-DNAs are substantially degraded over a period of hours to days - thereby seriously limiting their utility in antisense experiments running more than a few hours. Further, unpublished reports suggest that the thionucleotides resulting from degradation of S-DNAs are toxic to cells.

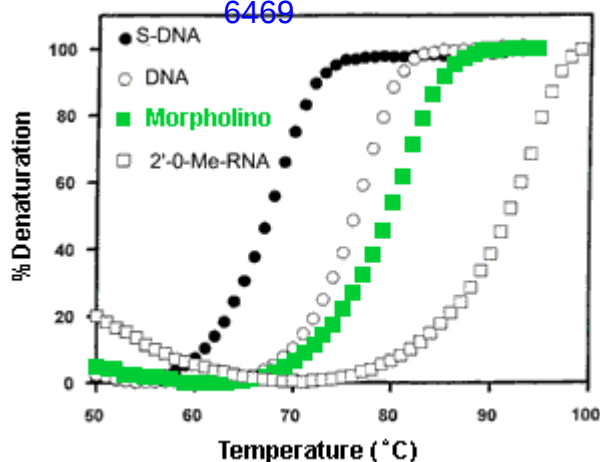
Chimeric oligos, which have recently become very popular, contain a segment of DNA or S-DNA (typically 8 to 12 bases) sufficient to afford RNase H cleavage of a paired target RNA. That RNase H-competent segment is flanked on one or both sides with an RNase H-independent segment designed to increase target binding affinity and/or increase resistance to exo nucleases. While chimeras have improved stability, it seems unlikely they will ever achieve satisfactory stability in biological systems because their RNase H-competent segment is inherently sensitive to endo nucleases within cells.

In sharp contrast to the limited stabilities inherent in the above RNase H-competent oligos (DNA, S-DNA, chimeras), Morpholino oligos have been shown to be completely resistant to nucleases, as well as being resistant to a broad range of other degradative factors in biological systems - even including a liver homogenate [Hudziak et al., 1996]. Accordingly, Morpholinos are effective in even long term experiments and they are free of complications which could arise from toxic degradation products.

## 3. Binding Affinity

The thermal melt ( $T_m$ ) results in Figure 4 illustrate the affinity of a Morpholino oligo (comprising A, C, G and U bases) for its complementary RNA sequence, as well as the affinities of corresponding S-DNA, DNA, and 2'O-Methyl RNA oligos for that same RNA sequence. As seen in this figure, the S-DNA has by far the lowest affinity, DNA has the next higher affinity, Morpholino a still higher affinity, and 2'O-Methyl RNA has the highest affinity. It should be noted that a corresponding PNA (Peptide Nucleic Acid)/RNA duplex would have a  $T_m$  quite similar to that of the 2'O-Methyl RNA/RNA duplex.

Figure 4. Thermal Melt Profiles For Various Oligo/RNA Duplexes



In 1998 **GENE TOOLS**, the sole commercial producer of research quantities of Morpholinos, switched from uracils to thymines in the production of Morpholinos. This increases the  $T_m$  a few degrees over that shown in Figure 5 and significantly increases efficacy. A "new" 25-mer containing thymines has comparable efficacy to a corresponding "old" 28-mer containing uracils. Switching from uracils to thymines also increases the Morpholino's ability to invade RNA secondary structure - thereby further improving its already outstanding targeting predictability.

#### 4. Efficacy: Cell-Free

It has been found that in a cell-free translation system with added RNase H (required for good S-DNA activity) the "old" 25-mer Morpholinos containing uracils typically exhibited slightly higher efficacies than corresponding S-DNAs, while "new" thymine-containing 25-mer Morpholinos generally achieve substantially higher efficacies than corresponding S-DNAs.

In contrast to the case for low-affinity S-DNAs, Figure 5 shows that the 2'-O-Methyl RNA/RNA duplex has an appreciably higher  $T_m$  than the corresponding Morpholino/RNA duplex. From this one might predict that 2'-O-Methyl RNAs should have greater efficacies than corresponding Morpholinos. However, experiments suggest that in cell-free translations Morpholinos generally have slightly higher efficacies and substantially better specificities than corresponding 2'-O-Methyl RNAs [[ANTIVIRALS Inc. Technical Report 3, 1993](#)].

The  $T_m$  values for PNA/RNA duplexes closely resemble the high  $T_m$  values for corresponding 2'-O-Methyl RNA/RNA duplexes. However, contrary to the case for 2'-O-Methyl RNAs, in cell-free studies with equal length PNAs and Morpholinos (18-mers) the higher-affinity PNAs generally achieve moderately higher efficacies than the corresponding lower-affinity Morpholinos. However, this efficacy advantage of PNAs is counterbalanced by the Morpholinos' substantially better sequence specificities.

In a more realistic analysis where the longest-commercially-available PNAs (18-mers) are compared to corresponding longest-commercially-available Morpholinos (25-mers), the longer Morpholinos achieve much higher efficacies than the shorter PNAs. Contrary to conventional wisdom which holds that specificity decreases with increasing length, the 25-mer Morpholinos also afford substantially higher sequence specificities than the 18-mer PNAs. Finally, the higher-efficacy/higher-specificity 25-mer Morpholinos are less expensive than the lower-efficacy/lower-specificity 18-mer PNAs.

#### 5. Efficacy: In Cells

In experiments with Morpholinos carried out at ANTIVIRALS Inc. it has been found that Morpholino antisense oligos which exhibit good activity in a cell-free translation system also exhibit correspondingly good activity when scrape-loaded into cultured animal cells [submitted for publication]. Similar correspondence between cell-free and in-cell activities of Morpholinos has been reported by Kobzik and coworkers at Harvard [[Taylor et al., 1996](#)] and by Kole and coworkers at Univ. of

North Carolina [Ryszard Kole, personal communication], and by Giles at University of Liverpool.

A number of S-DNAs previously shown to have excellent efficacies in a cell-free test system were also tested for their efficacies within cells. In sharp contrast to the high in-cell efficacies of Morpholinos, the scrape-loaded S-DNAs typically showed only slight in-cell efficacy, and then only at concentrations hundreds of times higher than required for good activity by Morpholinos.

Initially it was suspected that lack of in-cell activity by scrape-loaded S-DNAs might be due to their multiple negative backbone charges preventing good cell entry during the scrape-load procedure. However, when fluorescein-labeled oligos were scrape-loaded into cells it was seen that this procedure achieves delivery of S-DNAs as well or better than delivery of Morpholinos [Summerton et al., 1997].

A possible explanation for the very poor in-cell efficacy we've seen with S-DNAs is that their complexes with target sequences are disrupted by some cellular factor. In this regard a factor in the nucleus has been reported which destabilizes RNA secondary structure [Pontius & Berg, 1992] and an RNA helicase has been reported which strand separates DNA/RNA duplexes [Flores-Rozas & Hurwitz, 1993]. Moulds et al. at Gilead Sciences have postulated that some such strand separating factor acts on antisense oligo/RNA duplexes within cells [Moulds et al., 1995]. This is based on experiments wherein they paired high-affinity anionic antisense oligos with their respective target RNAs and microinjected these duplexes into cells. They found that the oligos were efficiently stripped off their RNAs - evidenced by translation of the protein coded by that RNA.

It is postulated that this cellular strand-separating factor may strip off anionic S-DNAs but fail to act on corresponding oligo/RNA duplexes when the oligo is non-ionic - as is the case for Morpholinos. The premise is that most proteins which interact with nucleic acids do so in substantial part via ionic interactions with the anionic backbones of nucleic acids. Thus the strand-separating factor may simply lack an effective handle for interacting with the non-ionic Morpholino antisense oligos in oligo/RNA duplexes.

An additional contribution to the excellent in-cell efficacies of Morpholinos and the poor in-cell efficacies of S-DNAs in scrape-loaded cells may derive from their respective subcellular localization. Specifically, when low concentrations of fluorescein-labeled S-DNAs are scrape-loaded into cells fluorescence microscopy shows them to be largely sequestered in the nucleus. In contrast, fluorescein-labeled Morpholinos scrape-loaded into cells are distributed throughout the cytosol and nucleus.

To appreciate the impact these subcellular distributions may have on efficacy it should be noted that in the nucleus transcription of a pre-mRNA, processing of that pre-mRNA, and transport of the mature mRNA to the cytosol typically occurs within minutes. In contrast, translation of the mRNA in the cytosol typically continues for hours to days. As a consequence, S-DNAs sequestered in the nucleus may have access to their target RNAs only in the brief time between transcription and export to the cytosol, while the more homogeneously distributed Morpholinos should have access to their target RNAs during the RNAs' brief sojourn in the nucleus and during their far longer residence in the cytosol. It seems quite possible that this large difference in target access time might contribute substantially to the high in-cell efficacies of Morpholinos and the low in-cell efficacies of S-DNAs.

[Table of Contents](#)

---

## Specificity

A key factor which lured many scientists (and investors) into the antisense field was the expectation that an antisense therapeutic could, with near perfect specificity, block its targeted genetic sequence while exerting essentially no other effects on the patient [Summerton, 1979]. It was hoped such exquisite specificity by antisense therapeutics would avoid the severe toxicities characteristic of present small-molecule antiviral and anticancer therapeutics. These great expectations largely died for many scientists in the antisense field, particularly those in pharmaceutical research, when: a) it became widely (but erroneously) believed that RNase H-competency was essential for good efficacy; and, b) the severe specificity limitations of S-DNAs became widely appreciated.

While the challenges to developing antisense therapeutics which are both highly effective and highly specific are daunting, success in meeting these and other challenges may lead to safe and effective treatments for a broad range of currently intractable and devastating diseases.

To meet the specificity challenge antisense oligos should have a "minimum inhibitory length" (MIL) sufficiently long to avoid attack on essentially all nontarget sequences in the cellular RNA pool. The MIL is defined as the shortest length of oligo of a given structural type which achieves substantial target inhibition at concentrations typically achieved in the cytosol/nuclear compartment of treated cells. For antisense therapeutics in humans the RNA pool includes all of the RNA species in all of the different tissues of the patient. This is estimated to be about 200 million bases of unique-sequence RNA, based on about 6% to 7% of the genome being transcribed in humans post embryogenesis.

The challenge for RNase H-competent oligos (ie., DNA, S-DNA, and chimerics) is to distinguish a single selected target sequence from this vast pool of RNA sequences.

Happily, the challenge for RNase H-independent oligos, such as Morpholinos, is less daunting. This is because RNase H-independent types are inactive when targeted against the approximately 95% to 98% of cellular RNA sequences comprising intron sequences in pre-mRNAs and amino acid-coding and 3'untranslated sequences more than about 20 bases 3' to the translational start site in mRNAs. As a consequence, Morpholinos should enjoy an inherent 20-fold to 50-fold specificity advantage over S-DNAs and chimerics because Morpholinos are only required to distinguish their target sequences from about 2% to 5% of the sequences comprising the patient's RNA pool.

Table 1 illustrates the importance of an adequate MIL value for achieving good sequence specificity. This table tabulates MIL values and the corresponding number of inherent cellular RNA sequences expected to be inadvertently inactivated by 25-mer oligos of the two fundamental antisense classes: RNase H-competent (eg., S-DNAs and chimerics) and RNase H-independent (eg., Morpholinos and PNAs). In calculating the values in this table an estimated RNA pool size of 200 million unique-sequence bases was used, as well as an estimate that sequences targetable by RNase H-independent oligos comprise 5% of that RNA pool.

Table 1. Estimated numbers of inadvertent targets for a 25-mer oligo in the human RNA pool as a function of MIL

MIL	RNase H Competent	RNase H Independent
7	232,000	11,600
8	55,900	2,750
9	13,000	648
10	3,050	153
11	715	36

12	167	8
13	38	2
14	9	0
15	2	0
16	0	0

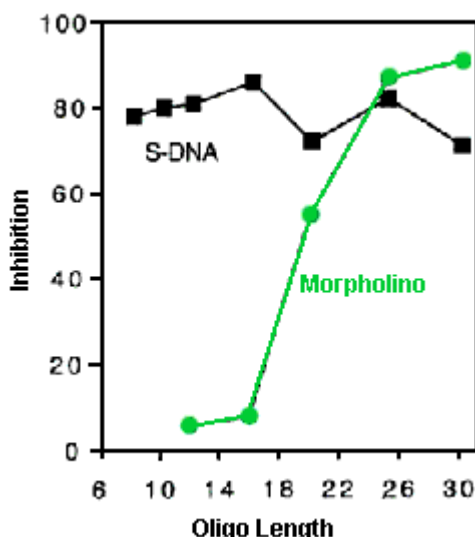
$$\text{Inadvertent targets} = \frac{\text{pool complexity}}{4^{\text{MIL}}} \times (\text{oligo length} - \text{MIL} + 1)$$

Pool Complexity: 200,000,000 for RNase H-competent.  
10,000,000 for RNase H-independent.

The values in this table suggest that to achieve very high sequence specificity an RNase H-competent 25-mer, such as an S-DNA, may need an MIL as high as 15 to 16. In contrast, to achieve the same high specificity a corresponding RNase H-independent oligo, such as a Morpholino, needs an MIL of only 13 to 14.

Experiments have been carried out at ANTIVIRALS Inc. (now [Sarepta Therapeutics](#)) to estimate MIL values for both RNase H-competent S-DNAs and RNase H-independent Morpholinos. In these activity versus length experiments both S-DNA and Morpholino oligos ranging in length from 8 bases to 30 bases, all targeted against the same region of rabbit alpha-globin leader sequence, were assessed in a cell-free translation system for their abilities to inhibit translation of a downstream luciferase coding sequence [for specifics on this test system see: [Summerton et al., 1997]. Figure 5 shows the respective translational inhibition values at oligo concentrations of 300 nanoMolar.

Figure 5. Activity Versus Oligo Length In Cell-free Translation System With 300 nM Oligo



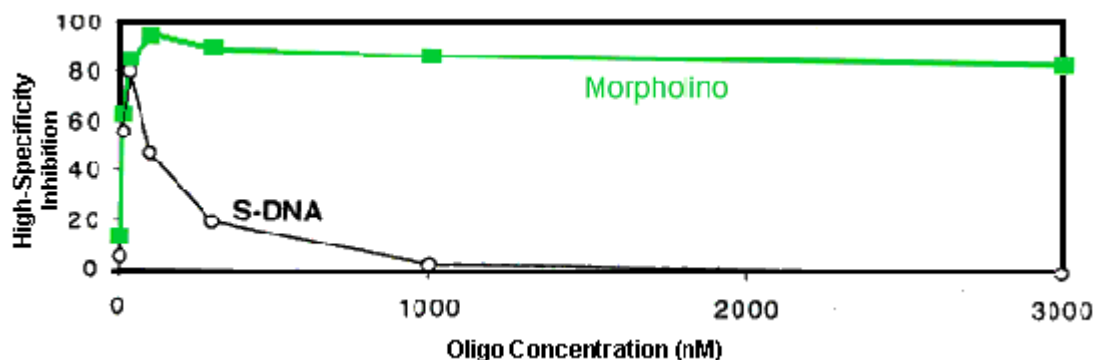
The results in Figure 5 suggest that under the conditions of this experiment S-DNAs have an MIL value of less than 8. This is far short of the minimum of 15 to 16 estimated to be required for high sequence specificity by RNase H-competent oligos. In sharp contrast, Morpholinos in this experiment have an MIL value of about 16. This is comfortably over the minimum of 13 to 14 expected to be required for high sequence specificity by RNase H-independent oligos.

The values in Table 1 combined with the results in Figure 5 suggest that every S-DNA may have a large number of inadvertent targets in human cells. In contrast, any given Morpholino is expected to have essentially no inadvertent targets.

To test these predictions, highly stringent specificity assays have been carried out in a cell-free translation

system. In these experiments two oligos of each structural type were used. One oligo of each type was perfectly complementary to its target mRNA to provide a measure of total inhibition achieved by that oligo type. The other oligo of that type incorporated 4 mispairs to that same target sequence, with the longest run of perfect pairing comprising 10 contiguous base pairs, to provide an emulation of the estimated level of sequence homology likely to be encountered in the RNA pool within human cells. The difference between these two inhibition values at each concentration, defined as "high-specificity inhibition", provides a concentration-dependent measure of the high-specificity inhibition achieved by that structural type. Results from a representative experiment are shown in Figure 6 [experimental as in [Summerton et al., 1997].

Figure 6. High-Specificity Inhibition In Cell-free Translation System



As predicted from the MIL values in Table 1 and the length versus activity results in Figure 12, the results in Figure 6 demonstrate that the RNase H-competent S-DNA achieved little high-specificity inhibition (47% to 80%) and then only in a quite narrow concentration range (10 nanoMolar to 100 nanoMolar). In contrast, but also in accord with these predictions, the RNase H-independent Morpholino achieved excellent high-specificity inhibition (84% to 95%) over a vastly broader concentration range (30 nanoMolar to well over 3,000 nanoMolar).

These results suggest that the long sought "exquisite specificity" goal of antisense has been achieved by Morpholinos. In contrast, because of the basic mechanism underlying RNase H-competency it is unlikely RNase H-competent oligos (S-DNAs and chimerics) can ever achieve a commensurate level of specificity.

[Table of Contents](#)

## Commercial Source

**GENE TOOLS, LLC** markets research quantities of Morpholino oligos worldwide. Our website has information on Morpholino [products](#), [prices](#), and [ordering](#).

You can [contact us](#) electronically or by using the information at the bottom of every page on this site.

In Japan, [Funakoshi Co.](#) is the distributor of Morpholino oligos and related products.

[Table of Contents](#)

## References

1. ANTIVIRALS Technical Report 3 (1993). Comparative studies of efficacy and specificity of translation inhibition in reticulocyte lysates. Antisense Res. Dev. 3 , following p. 306.
2. Flores-Rozas & Hurwitz (1993). Characterization of a new RNA helicase from nuclear extracts of HeLa cells which translocates in the 5' to 3' direction. J. Biol. Chem. 268 , 21372.
3. Hudziak, Barofsky, Barofsky, Weller, Huang, Weller (1996). Resistance of Morpholino phosphorodiamidate oligomers to enzymatic degradation. Antisense Nucleic Acid Drug Dev. 6 , 267.
4. Kang, Cho, Johnson, Weller, Huang, Summerton (1992). Stacking interactions of ApA analogues with modified backbones. Biopolymers 32 , 1351.
5. Moulds, Lewis, Froehler, Grant, Huang, Milligan, Matteucci, Wagner (1995). Site and mechanism of antisense inhibition by C-5 propyne oligonucleotides, Biochem. 34 , 5044.
6. Pontius & Berg (1992). Rapid assembly and disassembly of complementary DNA strands through an equilibrium intermediate state mediated by A1 hnRNP protein. J. Biol. Chem. 267 , 13815.
7. Stirchak, Summerton & Weller (1987). Uncharged stereoregular nucleic acid analogues. 1. Synthesis of a cytosine-containing oligomer with carbamate intersubunit linkages. J. Organic Chemistry 52 , 4202.
8. Summerton (1989). Uncharged nucleic acid analogs for therapeutic and diagnostic applications: oligomers assembled from ribose-derived subunits. In: Discoveries in Antisense Nucleic Acids; Ed.: C. Brakel; Publisher: The Portfolio Publishing Co., Woodlands, Texas; pages 71 - 80.
9. Summerton & Weller (1997a). Morpholino antisense oligomers: design, preparation and properties. Antisense Nuc. Acid Drug Dev. 7 , 187.
10. Taylor, Paulauskis, Weller, Kobzik (1996). In vitro efficacy of Morpholino-modified antisense oligomers directed against tumor necrosis factor-alpha mRNA. J. Biol. Chem. 271, 17,445.

## Online Store

Ready to order?

[Gene Tools Store](#)

Gene Tools, LLC  
1001 Summerton Way  
Philomath, OR 97370 USA

Ph.: (541) 929-7840 | Fax: (541) 929-7841

[Contact Us](#)

# Exhibit 40



## Review

## Towards Personalized Allele-Specific Antisense Oligonucleotide Therapies for Toxic Gain-of-Function Neurodegenerative Diseases

Jacob Helm <sup>1,2,3</sup> , Ludger Schöls <sup>1,2</sup> and Stefan Hauser <sup>1,2,\*</sup> <sup>1</sup> German Center for Neurodegenerative Diseases (DZNE), 72076 Tübingen, Germany<sup>2</sup> Hertie Institute for Clinical Brain Research and Department of Neurology, University of Tübingen, 72076 Tübingen, Germany<sup>3</sup> Graduate School of Cellular and Molecular Neuroscience, University of Tübingen, 72076 Tübingen, Germany

\* Correspondence: stefan.hauser@dzne.de

**Abstract:** Antisense oligonucleotides (ASOs) are single-stranded nucleic acid strings that can be used to selectively modify protein synthesis by binding complementary (pre-)mRNA sequences. By specific arrangements of DNA and RNA into a chain of nucleic acids and additional modifications of the backbone, sugar, and base, the specificity and functionality of the designed ASOs can be adjusted. Thereby cellular uptake, toxicity, and nuclease resistance, as well as binding affinity and specificity to its target (pre-)mRNA, can be modified. Several neurodegenerative diseases are caused by autosomal dominant toxic gain-of-function mutations, which lead to toxic protein products driving disease progression. ASOs targeting such mutations—or even more comprehensively, associated variants, such as single nucleotide polymorphisms (SNPs)—promise a selective degradation of the mutant (pre-)mRNA while sparing the wild type allele. By this approach, protein expression from the wild type strand is preserved, and side effects from an unselective knockdown of both alleles can be prevented. This makes allele-specific targeting strategies a focus for future personalized therapies. Here, we provide an overview of current strategies to develop personalized, allele-specific ASO therapies for the treatment of neurodegenerative diseases, such as Huntington's disease (HD) and spinocerebellar ataxia type 3 (SCA3/MJD).

**Keywords:** antisense oligonucleotide; ASO; neurodegenerative diseases; toxic gain-of-function; allele-specific targeting; SNP



**Citation:** Helm, J.; Schöls, L.; Hauser, S. Towards Personalized Allele-Specific Antisense Oligonucleotide Therapies for Toxic Gain-of-Function Neurodegenerative Diseases. *Pharmaceutics* **2022**, *14*, 1708. <https://doi.org/10.3390/pharmaceutics14081708>

Academic Editors: Tina Kausse and Philippe Barthélémy

Received: 24 June 2022

Accepted: 12 August 2022

Published: 16 August 2022

**Publisher's Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Copyright:** © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

## 1. Introduction

Antisense oligonucleotides (ASO) are short, modified single-stranded DNA, RNA, or hybrid DNA-RNA sequences that bind complementary cellular RNAs, such as (pre-)mRNAs, or noncoding RNAs, such as microRNAs, thereby influencing their further processing. In the central dogma of protein synthesis, such an ASO–(pre-)mRNA interaction would prevent the translation of a potentially toxic gene variant. Continuous new modifications of ASOs at their sugar, backbone, or base aim to improve pharmacokinetics and dynamics (reviewed in [1–5]). Thereby, it becomes possible to develop ASOs that target the (pre-)mRNA in an allele-specific manner. This can be achieved by, e.g., selectively targeting single nucleotide polymorphisms (SNP) on the mutant allele, thus providing an approach for a personalized allele-specific treatment [6,7].

Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), Alzheimer's disease (AD) and spinocerebellar ataxia (SCA) are neurodegenerative diseases in which a toxic version of a protein can lead to abnormal protein aggregation [8–10]. In such cases, an altered sequence or post-translational modification leads to a conformational or structural change and an increased risk for aggregation and mislocalization [11–14]. Even though the cause of several monogenetic neurodegenerative diseases driven by toxic

gain-of-function mechanisms has known for years or decades [15–18], there is so far no cure, and current treatments only mitigate specific symptoms, but do not stop or slow down the progression of the disease [19]. One reason why no suitable therapy options have been found so far might be that the toxic gain-of-function often leads to a misprocessing of the protein, which manifests as an aggregation that impairs multiple cellular pathways, leading to neurotoxic effects [11,20,21].

In monogenetic disorders with dominant inheritance, a genetic diagnosis can potentially be made early in life, even before a clinical manifestation of the disease. This offers, in principle, the chance to prevent disease manifestation, if treatment becomes available that “silences” the toxic gain-of-function mutations before the disease-causing effects can no longer be offset.

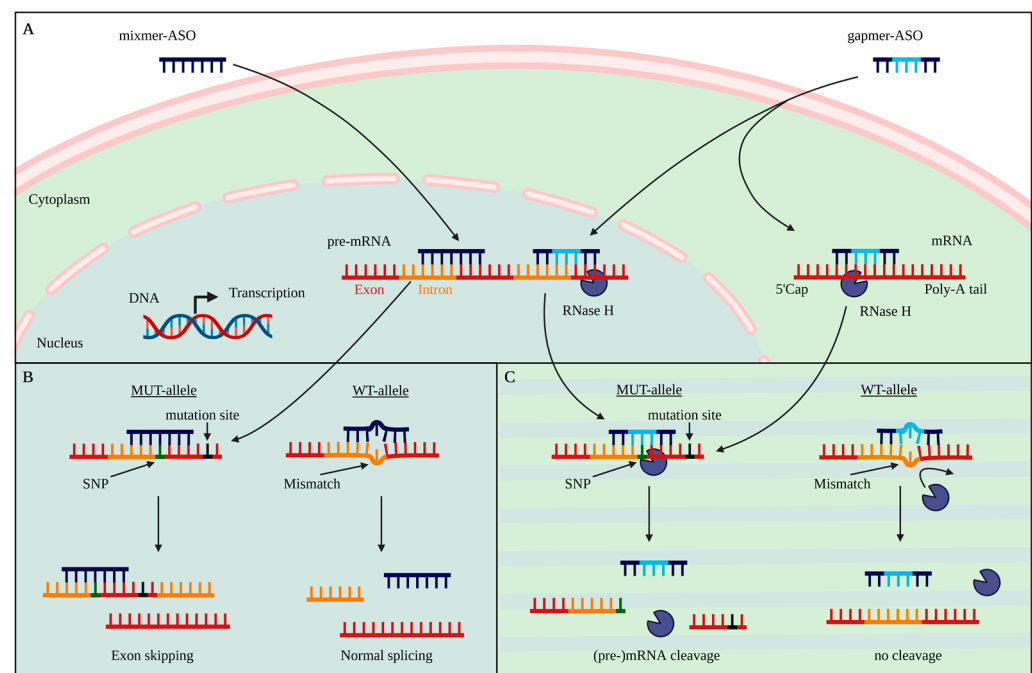
One possibility to develop a causal therapy could be to degrade the disease protein. This could ideally involve strategies to selectively degrade the toxic protein variant while preserving the wild type form of the protein. ASOs could play a key role in such a therapeutic strategy. In autosomal dominant inherited neurodegenerative diseases with toxic gain-of-function mutations, minor differences between wild type and pathogenic alleles (e.g., SNPs) could be used to selectively degrade the mutant (pre-)mRNA and by this, prevent expression of the toxic protein variant while preserving expression of the healthy copy from the wild type allele [6,7]. Here, we describe the current state and possibilities of personalized, allele-specific research and treatment in neurodegenerative diseases caused by toxic gain-of-function mutations.

## 2. Antisense Oligonucleotides

Antisense oligonucleotides are composed of a strand of DNA or RNA as an initial structure that binds via Watson–Crick hybridization to its complementary RNA strand. The internalization of ASOs is not necessarily linked to a single pathway. Uptake is mostly receptor-dependent with subsequent intracellular trafficking from early endosomes to late endosomes, Golgi apparatus, and lysosomes. However, to fulfill its function in the cytoplasm and nucleus, the ASO must escape the endosome in a process that is likely associated with endosomal transport proteins [22,23]. A similar transport capability for ASOs is not described for the blood–brain barrier (BBB), making it possible to specifically target the brain by intrathecal application [3]. Length and nucleotide arrangement define the mode of action, as well as the target specificity. The arrangement of nucleotides can be a modified RNA strand, a mixmer design, with various alignments of DNA and RNA nucleotides, or a gapmer design, with a central DNA area that is flanked by RNA-based nucleotide wings [24–27]. Generally, two modes of action can be distinguished (Figure 1A). In the first, modified RNA strands and mixmers act as a steric block. They bind to their target pre-mRNA and thus prevent further processing or modulate splicing [28] (Figure 1B). Binding to complementary pre-mRNA at splice sites can lead to either exon skipping or exon inclusion. Therefore, ASOs can specifically be used to generate a spliced mRNA lacking exons, resulting in frameshifts, mismatches, or early stop codons leading to nonsense-mediated mRNA decay (NMD). In addition, deep intronic splice variants and non-productive transcripts that are pathologically integrated can be targeted by this approach [3,29,30]. The second mode of action of ASOs is via RNase H-dependent degradation. For this, gapmers consisting of flanking RNA wings with a central DNA portion are mainly used. Binding of DNA to its target (pre-)mRNA in the nucleus or cytoplasm recruits RNase H and leads to the degradation of the complementary counterpart [3,30–33], (Figure 1C). By modifying the backbone, sugar, and/or base of the designed ASO, its characteristics, including stability, longevity, toxicity, target affinity, and specificity, can be modulated (Table 1).

**Table 1.** Antisense oligonucleotide modifications and properties.

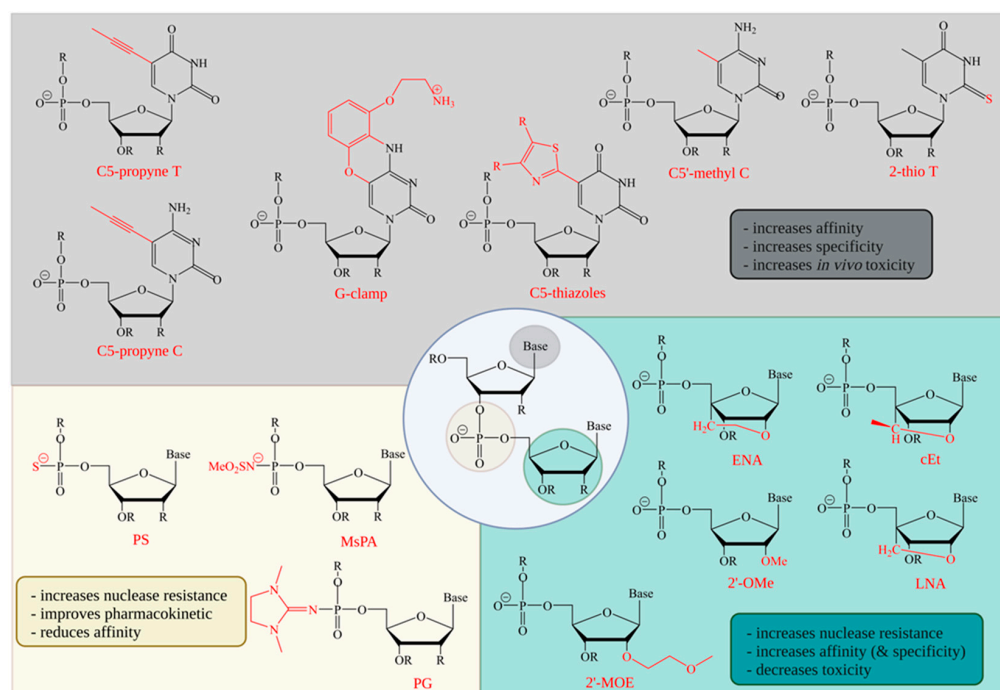
Modification	T <sub>m</sub> per Nucleotide	References	Specific	General
<b>Backbone</b>				
Phosphorothioate (PS)	0.45 to 1 °C	[27,34–39]	<ul style="list-style-type: none"> <li>Increases toxicity</li> <li>Improves RNase H recognition</li> </ul>	
Mesylphosphoramidate (MsPA)	1.3 to +1.1 °C (with respect to PS)	[39,40]	<ul style="list-style-type: none"> <li>Reduces toxicity</li> <li>Number and position influences RNase H activation</li> <li>Incorporation in gap reduces protein binding</li> <li>Nuclease resistance (MsPA &gt; PS)</li> </ul>	<ul style="list-style-type: none"> <li>Increases nuclease resistance</li> <li>Improves pharmacokinetics</li> <li>Reduces affinity</li> <li>Stereoisomers</li> </ul>
Phosphoryl guanidines (PG)	1.2 to 0 °C	[41–44]	<ul style="list-style-type: none"> <li>Reduces toxicity</li> <li>Prevents RNase H binding, when used in DNA-gap</li> <li>Nuclease resistance (PG &gt; PS)</li> <li>Reduced cellular uptake compared to PS</li> </ul>	
<b>Sugar</b>				
Locked nucleic acids (LNA)	+1.5 to +9.1 °C	[27,45–51]	<ul style="list-style-type: none"> <li>Can improve specificity</li> </ul>	
2'-O-methyl (2'-OMe)	0 to +1.3 °C	[27,34,38,46]		
2'-O-methoxyethyl (2'-MOE)	+0.9 to +1.9 °C	[34,46,51–53]	<ul style="list-style-type: none"> <li>Improves cellular uptake</li> </ul>	<ul style="list-style-type: none"> <li>Increases affinity</li> <li>Increases nuclease resistance</li> <li>Decreases toxicity</li> <li>Not compatible with DNA-gap</li> </ul>
2'-4'-constrained 2'-O-ethyl (2'-cEt)	+4.7 to +6.1 °C	[51,54]	<ul style="list-style-type: none"> <li>Can improve specificity</li> <li>Exonuclease resistance cET &gt; LNA</li> <li>Stereoisomers</li> <li>Can increase specificity</li> </ul>	
2'-O,4'-C-ethylene-bridged nucleic acid (ENA)	+5.2 °C	[49]	<ul style="list-style-type: none"> <li>Exonuclease resistance ENA &gt; LNA</li> </ul>	
<b>Base</b>				
G-clamp	+4 to +18 °C	[46,55,56]	<ul style="list-style-type: none"> <li>Can improve specificity</li> <li>Reduces RNase H activity</li> </ul>	
C5-propyne C	+1.5 to 1.6 °C	[55,57–59]	<ul style="list-style-type: none"> <li>Reduces RNase H activity</li> <li>Increases resistance</li> </ul>	
C5-propyne T	+0.9 to +2.6 °C	[34,54,55,57–59]	<ul style="list-style-type: none"> <li>Reduces RNase H activity</li> <li>Increases resistance</li> </ul>	<ul style="list-style-type: none"> <li>Increases affinity</li> <li>Can induce toxicity</li> </ul>
2-thio-thymidine	+0.3 to +1.8 °C	[54,60]	<ul style="list-style-type: none"> <li>Can improve specificity</li> </ul>	
5'-thiazole analogues	+1.7 to 2.2 °C	[58,59]	<ul style="list-style-type: none"> <li>RNA affinity (thiazole &gt; propyne)</li> </ul>	
5-Methyl cytosine	0 to +1.1 °C	[25,34]		



**Figure 1.** Mode of action of allele-specific antisense oligonucleotide strategies. (A) Mixmer ASOs and gapmer ASOs can be transported through cell membranes and nuclear pores to target (pre-)mRNA. (B) Mixmers act as a steric block that binds to the target pre-mRNA and can modulate the splicing process. Steric block ASOs can be used to selectively target the SNP region and therefore, modulate splicing. Binding to the WT allele has a lower affinity compared to the MUT allele, due to a mismatch. This results in a less effective splicing modulation. (C) Gapmer ASOs can either bind intronic regions or exonic regions of (pre-)mRNA in the nucleus or cytoplasm. Binding of gapmers with their target (pre-)mRNA recruits RNase H. The DNA:RNA binding leads to a degradation of the (pre-)mRNA. SNPs that appear on the same allele as the disease-causing mutation, such as a CAG repeat expansion, can be directly targeted with an ASO, potentially leading to a mutant-specific degradation. The mismatch on the WT allele results in a conformational change that leads to a reduced cleavage capacity.

### 3. Backbone Modifications

The most common modification of the backbone is a replacement of a phosphodiester (PO) with a phosphorothioate (PS) bond (Figure 2). This increases nuclease stability and cellular uptake; in addition, the interaction with albumin allows the ASO to be transported within the cerebrospinal fluid (CSF) or blood plasma [61–63]. ASO–protein bindings are particularly important with regard to pharmacokinetic properties, since these interactions can delay renal clearance [64]. Since this clearing mechanism is independent from the brain, the main focus on backbone modifications of ASOs for treatments of the central nervous system is on the improved uptake and transport associated with protein binding [61]. The interaction with intracellular proteins is potentially associated with an increase in toxicity, which can be reduced by a site-specific introduction of a neutral PO linkage at position 2 or 3 in the DNA gap [35,36]. However, this modification also results in a reduction of affinity, with a reduction in melting temperature of  $-0.45$  to  $-1$  °C per nucleotide [34]. Another aspect of a sulfur modification is that the negative charge is exclusively on the sulfur atom. This leads to different possible chiralities between the phosphorus and the sulfur atoms [65]. The effects of stereoisomerism of PS on the stability and target cleavage site are currently under debate.



**Figure 2.** Common ASO nucleotide modifications. ASOs can be optimized at different positions of the nucleotide. Modifications at the phosphate (yellow) reduce affinity, but have positive effects on immunoreactivity and nuclease resistance and thus, also on longevity. Sugar modifications (turquoise) can increase nuclease resistance, affinity, and specificity and decrease toxicity. Disadvantageously, 2'-O modifications cause the loss of deoxyribose character and cannot be recognized by RNase H. Therefore, these modifications cannot be implemented in the DNA gap region of gapmer ASOs. Base modifications (gray) may have the greatest potential for increasing the specificity of individual bases, but are also associated with an increased toxicity *in vivo*. cET = constrained ethylbridged nucleic acid; ENA = 2'-O,4'-C-ethylene bridged nucleic acid; LNA = locked nucleic acid; MsPA = mesyl phosphoramidate; PG = phosphoryl guanidine; PS = phosphorothioate; 2'-MOE = 2'-O-methoxyethyl; 2'-OMe = 2'-O-methyl.

RNase H interacts with the DNA:RNA junction at three consecutive nucleotides. Iwamoto et al. proposed a stereochemical PS backbone of 3'-SpSpRp-5' in the center of the DNA gapmer as the most suitable site, with the cleavage site located two nucleotides upstream from the Rp with respect to the DNA [66]. This pattern and its capabilities could not be confirmed by Østergaard and colleagues. However, they showed that a single Rp junction at position 7 in a Sp DNA gap, resulted in a single cleavage site, located two nucleotides downstream on the DNA [37]. The potential to predefine cleavage sites could increase the focus for nucleotide-specific modifications to specific positions, thus increasing the impact of modifications at specific sites.

A relatively novel modification of the backbone is the use of mesylphosphoramidate (MsPA) [67,68]. Stetsenko et al. described positive effects on RNase H activity when DNA-ASOs were modified with MsPA compared to PS-modified DNA-ASOs [39]. Ionis Pharmaceuticals could not confirm these positive effects when DNA gapmer ASOs were uniformly modified with MsPA. Slight improvement in RNase H activity could be observed when a maximum of 5 PS bonds in the DNA gap were replaced by MsPA, or Gap 2'-OMe PS ASOs were modified with MsPA at two positions in the 3' wing, or the 5' DNA gap [40,69]. Additionally, MsPA positively influences toxicity and half-life of ASOs. An improvement in the stability of nucleases could be obtained when 4 PS bonds of the DNA gap were replaced by MsPA. Even more important might be the effect of MsPA on the interactions with proteins. These interactions could already be substantially reduced by 2 MsPA instead of PS in the DNA gap and could thus become particularly important if

protein-associated toxicity needs to be reduced [39,40]. A recent publication indicates the interaction between the design of ASOs and the ionotropic glutamate  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA). It has been shown that an increasing number of G bases in an ASO gapmer increases the risk of AMPAR-associated neurotoxicity [70]. Therefore, it will be interesting to see if MsPA modifications around the Gs have an impact on the interaction with AMPAR, thereby modifying potential toxicity. In vivo studies showed a superior effect of MsPA over PS in a fully modified ASO, when delivered in folate-containing liposomes. MsPA showed an improved tumor tissue penetration and therefore, improved action and retention of tumor growth, by targeting miR-21 [71].

Recent studies suggest that the understanding of stereochemical effects and further modifications, such as by using phosphoryl guanidine diester (PG), can improve the pharmacological effects [72–74]. PG was also incorporated into the backbone of an ASO in a recent HD trial (NCT05032196) [75]. The modification itself was already introduced in an oligonucleotide in 2014 [76]. PGs have a strong nuclease resistance and a reduced toxicity, but also an increased reduction in affinity and a loss of RNase H recognition, when present in DNA gaps. With their good nuclease resistance, it might be beneficial to use this modification at the flanking regions of an ASO [41–43]. Used in steric block ASOs, PGs can improve the activity and uptake of the corresponding ASO and suppress target gene expression [44].

#### 4. Sugar Modifications

Modifications of the sugar of a nucleotide are mostly accompanied by a modification at the 2' C atom or a total loss of the ring structure. In both cases, the molecular character of the DNA is modified to an extent that it is not recognized by RNase H [77–79] (Figure 2). Therefore, such modifications are not possible in the central DNA portion of a gapmer. In general, sugar modifications in steric block ASOs, or the flanking RNA part of gapmers, improve nuclease resistance and RNA affinity [31,51]. The idea of improving the affinity of antisense strands to their target is already decades old. In 1987, Inoue et al. used a 9-nucleotide-long gapmer with two or three flanking 2'-O-methylnucleotides in which the RNase H cleavage site was specifically located in the unmodified, central DNA [79]. This modification was also part of the first approved aptamer drug, pegaptanib, in 2004 [80]. Binding to RNA, this modification has minor effects on affinity, increasing melting temperature by 0 to +1.3 °C per nucleotide [27,34,38,46].

The 2'-O-methoxyethylnucleotide (2'MOE) modifications are more widely used. The 2'-MOE modifications have an even higher affinity than other modifications, as well as a good nuclear resistance, with an increase in melting temperature of +0.9 to +1.9 °C per nucleotide [34,46,52,53,81]. One of the most prominent uses of 2'MOE in ASOs is in nusinersen (Spinraza), which was FDA approved in 2016. Nusinersen is an end-to-end 2'MOE modified, 18-nucleotide-long steric block ASO that is used for the treatment of spinal muscular atrophy (SMA) [82]. A similarly prominent ASO example in which 2'MOE modifications were applied, but with a different function, is in the development of tominersen as potential treatment for Huntington's disease (HD). The gapmer ASO, developed by Roche and IONIS Pharmaceuticals, consists of a 10-nucleotide-long DNA-gap and 5 flanking 2'-O-methoxyethylnucleotides on both wings. In March 2021, the phase III trial was stopped due to risk/benefit concerns; nonetheless, a reduction in huntingtin protein was achieved and is thus proof of the principle of using ASOs with mechanism of action via RNase H degradation [2,83,84].

In another group of sugar modifications, a linkage between the 2'-O and the 4'-C results in a C3'-endo sugar pucker of the furanose. The so-called locked nucleic acids (LNA) change the helix structure towards A-type, with respect to a DNA:RNA binding. This prevents the duplex from RNase H cleavage [85,86]. LNA:RNA bindings have a high affinity, with an increase of up to 9 °C per nucleotide with respect to DNA:RNA bindings [27,45–50]. In addition, LNA provide increased protection against endonuclease (nuclease P<sub>1</sub>) and 3' exonucleases (snake venom phosphodiesterase, SVPD) compared to

DNA [49,51]. Three LNA modified nucleotides, each at the 3' and 5' ends, increase the half-life of the respective gapmer in human serum by 10-fold compared to unmodified DNA oligonucleotides [27]. LNAs also exhibit increased potency compared to 2'MOE, although this is also associated with increased toxicity [87].

Like LNA, 2'4'-constrained 2'-O-ethyl (2'-cEt) are part of the bridged nucleic acids (BNA). The 2'-cEt have an additional methyl group at the connecting C-atom. Since the stereochemistry of the methyl group can vary in its connection to the C atom, they are referred to as enantiomers *S*-cEt and *R*-cEt [88]. Compared to LNAs, both analogs showed comparable thermal stability, potency, and increased nuclease stability against SVPD [26,51].

Modifications of the sugar are particularly good at increasing affinity and nuclease resistance. However, since they cannot be used in the DNA gap, their influence on the cleavage site and the specificity of individual bonds is limited.

## 5. Base Modifications

Base modifications may have the greatest potential to increase specificity of a single base, but they also present the greatest challenges. Although an adjustment can lead to an increase in the affinity of the base, it can also lead to an enhanced affinity for non-complementary bases, leading to an elevated likelihood of mismatches. The purpose of base modulation is to increase the specificity of the entire ASO, but most importantly, of a single base to its complementary counterpart. The smallest variants of the modification of bases are methylations, such as the 5'-methylation of cytidines (Figure 2). This methylation can reduce immunostimulatory effects, especially of CpG dinucleotides, and increase nuclease stability and thermal stability, with an increase of up to 1.1 °C per nucleotide [25,34,89]. C5'-methylations of cytidine and uracil are used throughout the molecule in some steric block ASOs, such as Spinraza, and RNase H-activating ASOs [3]. A slightly larger modification is C5'-propynyl thymine and cytosine. Compared to the single C5'-methylation, C5'propynyls increase thermal stability by +0.9 to +2.6 °C per nucleotide. Depending on their position and stereochemistry, they may even improve specificity [34,54,55,57–59]. The 5'thiazole pyrimidine analogues have similar characteristics for base stacking, and they improve thermal stability, with +1.7 to +2.2 °C per base [59].

A further step towards allele-selective oligonucleotide modifications could be achieved by 2'-thio pyrimidine modifications. Østergaard et al. showed that by incorporating a single 2'-thio deoxythymidine (2'-thio dT) into the DNA gap of an ASO, the affinity, but also the specificity, could be increased. For a HTT SNP-targeting ASO, the difference between melting temperatures was highest (5.6 °C) when the 2'-thio dT modification was at the position of the SNP [60]. Another well-studied pyrimidine analogue is the G-clamp, a cytosine that is scaled up to a phenoxazine with 9'-O-aminoethyl. The G-clamp and newer approaches, like G<sup>8AE</sup>-clamp, or guadino-G-clamp, improve binding affinity and specificity to guanine by making additional use of the Hoogsteen binding site, forming a fourth hydrogen bond to guanine [56,90–92]. By this, the melting temperature can be increased by +4 to +18 °C per nucleotide [46,56], especially when the G-clamp is flanked by a 5'-cytidine. In addition, the G-clamp has fluorescent properties and a 3'exonuclease resistance [56]. Increased specificity was shown in a DNA-ASO consisting of 10 nucleotides. Comparisons between 5'methylcytosine and G-clamp modifications resulted in an increase in thermal stability by 18 °C with the G-clamp. Although the melting temperature of all mismatch partners also increased compared with 5'-methylcytosine, the difference in melting temperature between match and mismatch was greatest for the G-clamp, suggesting increased specificity [55]. At the same time, the incorporation of a single G-clamp appears to have little or no effect on RNase H cleavage [46,55]. It remains to be shown if specific base modifications, like the G-clamp, can be used to improve SNP-based allele-specific targeting approaches.

## 6. Selective vs. Non-Selective Targeting Strategies

Dominant inherited diseases are often caused by heterozygous mutations, with a toxic gain-of-function. Non-selective targeting of the diseased gene would reduce both the toxic and the wild type variant and therefore, diminish the function of the protein. With a selective targeting of the mutated allele, the toxic protein load could be reduced, while preserving the physiological function of the wild type protein. Even though tominersen, a non-selective HTT ASO, showed dose-dependent reduction in HTT in the phase I/IIa trial [93], the phase III trial was stopped because of unfavorable effects outweighing potential benefits [84,94–96]. Previous animal studies suggested that the toxic effect of mHTT is greater than the impairment resulting from a total loss of HTT [97]. However, it is hard to predict the ideal knockdown efficiency for nonselective degradation strategies, since it is difficult to estimate the right balance between necessary reduction in toxic mHTT and a negative treatment response due to the loss of wtHTT [2]. Similarly, in another CAG repeat disease, SCA3, very promising non-allele-specific reduction in ATXN3 has been achieved in humanized SCA3 mice, as well as patient-derived hESCs [98–100]. Recently, SCA3 ASOs have just entered phase I (NCT05160558).

Table 2 gives an overview of current clinical trials using ASOs in neurodegenerative diseases caused by toxic gain-of-function mutations (reviewed in [8,101]). Treating these diseases with non-selective ASOs would result in a reduction in the mutated and wild type protein with the aim of decreasing the toxic effects. At the same time, potential protective and physiologically necessary functions of the wild type protein are also reduced. Even though some functions of proteins causing neurodegeneration are known, there are still uncertainties (Table 2). An unspecific knockdown of such a protein may have serious, unforeseeable consequences. Specifically targeting the mutant allele is supposed to result in a selective degradation of the toxic protein and preservation of the healthy allele. One of the most approachable uses of an ASO that aims for allele-specificity would be to target the mutation directly. In the case of HD or polyQ SCAs, this would be the expanded CAG repeat. An approach to circumvent the toxic effect of the expanded CAG repeat is alternative splicing. Toonen et al. masked splicing signals of the SCA3 pre-mRNA, resulting in a shortened protein version that bypasses the CAG repeat containing exon 10 and most parts of exon 11 due to an early stop codon. This modified protein showed beneficial effects on pathogenicity, with a detectable in vivo longevity of 2.5 months [102]. Even though the approach was specific for CAG toxicity, the effects were not purely allele-specific, since both alleles are targeted with these steric block ASOs. One way to directly eliminate allele-selective toxicity of the expanded CAG repeat is to use ASOs that directly target the CAG repeat at the (pre-)mRNA level. Since several proteins physiologically possess CAG repeats, a single ASO could be used for treating several different CAG repeat diseases. Likewise, the number of potential off-targets may increase, since such a CAG-specific ASO could potentially bind to several genes with CAG repeats. Evers et al. observed that such an ASO, with 21 nucleotides targeting the CAG repeat, showed allele-specific reduction not only in HTT, but also in other CAG repeat diseases, such as SCA1, 3, and DRPLA. No reduction was detected in potential CAG repeats containing off-targets, such as SCA2, ZNF384, or TATA box binding protein (TBP) [103]. Other publications with predominantly steric block ASOs show similar success in HD, SCA1, and SCA3, with dose-dependent, allele-specific reductions of up to 6.6-fold. It is believed that these steric block ASOs bind more efficient to the expanded CAG repeat containing mRNA compared to the shorter WT version, thus providing translation in an allele-specific manner [104–106]. A potential disadvantage of directly targeting the CAG repeat could be that every third nucleotide of the ASO that targets the CAG repeat is a guanine. Recent studies showed that in vitro and in vivo neurotoxicity is associated with the number and position of Gs within an ASO. The risk for a potential neurotoxicity increases with the number of Gs within the ASO sequence.

**Table 2.** Clinical trials of antisense oligonucleotides in toxic gain-of-function neurodegenerative diseases.

Disease 1 Target	Cellular Function	ASO	Phase	ASO Type/Modifications	Ref./Clinical Trial
HD-HTT	Brain development, involved in vesicle trafficking and recycling, cell division, ciliogenesis, autophagy, development [107]	Tominersen, IONIS-HTT <sub>Rx</sub>	Phase III halted (03/21)	Non-allele-specific, PS 2'-MOE	[2,84,108] NCT03842969
HD-HTT		WVE-003 (WVE-120101 & 120102: suspended)	Phase I/II	Allele-specific, PS stereopure	[2,72,109] NCT05032196
ALS/FTD-FUS	DNA/RNA metabolism [110]	Jacifusen/ION36	Phase III	Mutation-specific (p.P525L), PS 2'-MOE	[111–113] NCT04768972
ALS-SOD1	Antioxidant [114]	Tofersen/IONIS-SOD1 <sub>Rx</sub> (BIIB067)	Phase III	Non-allele-specific, PS 2'-MOE	[115,116] NCT02623699 NCT03070119
ALS/FTD-C9ORF72	Repeat in noncoding region [117]	IONIS-C9 <sub>Rx</sub> (BIIB078)	Phase I discontinued (03/22)	Non-allele specific, PS 2'-MOE	NCT03626012 NCT04288856
ALS/FTD-C9ORF72		WVE004	Phase I/II	Allele-specific (Targeting V1 and V3 transcript), PS PG stereopure	[118,119] NCT04931862
ALS/SCA2-ATXN2	RNA metabolism [120]	ION541 (BIIB105)	Phase I/II	PS 2'-MOE	[83,121] NCT04494256
AD/FTD-MAPT (TAU)	Stabilizing & promotion of microtubule assembly [122]	IONIS-MAPT <sub>Rx</sub> (BIIB080)	Phase II	PS 2'-MOE	[123]
SCA3-ATXN3	Deubiquitinase [120]	ION260 (BIIB132)	Phase I	Non-allele-specific, PS 2'-MOE	[100,121] NCT05160558
PD-LRRK2	Kinase involved in lysosomal processes, autophagy, mitophagy, vesicle trafficking [124]	ION859 (BIIB094)	Phase I/II	PS 2'-MOE	[125] NCT03976349
PD-SNCA	Presynaptic protein, involved in SNARE complex assembly [126]	ION464 (BIIB101)	Phase II	PS 2'-MOE	[127] NCT04165486
Alexander disease-GFAP	Intermediate filament [128]	Zilganersen, ION373	Phase II	Non-allele-specific, PS 2'-MOE	[129] NCT04849741 CAS2305355-56-8

A less strong association to neurotoxicity was shown for the distance from the 3'-end to the first guanine. In contrast, increasing the number of As in an ASO had positive effects on the risk of potential neurotoxicity [70].

Like CAG repeat disorders, ASOs were also tested targeting other repeat diseases, such as *C9ORF72* in ALS/FTD patient cells. Different ASOs were designed targeting either the *C9ORF72* repeat (GGGGCC) itself or exonic and intronic regions up- and downstream of the repeat. Experiments on patient-derived cells and in vivo experiments showed mainly positive and allele-specific effects [130,131]. Recent publications also confirmed the possibility of achieving effective reduction via the RNase H-mediated degradation of *C9ORF72*, targeting either the repeat or intronic sequences of the gene [73,132]. Treatment of a patient with C9-ALS/FTD showed a marked reduction in polyGP-DPR (dipeptide repeat protein), with no medical or neurological adverse effects [132].

Success in patients was also reported with ASO treatment of other genes in the ALS/FTD spectrum. Treatment of an ALS patient carrying the *FUS*<sup>P525L</sup> mutation with ION363 (jacifusen) resulted in a mutant-specific reduction of FUS protein and its aggregation, reaching clinical phase III trials (Table 2) [113]. Additionally, an ASO that targets superoxide dismutase 1 (*SOD1*) also reached phase III in clinical studies [115,130,133]. Clinical trials have also been initiated in Alzheimer's disease using ASOs targeting *MAPT*. Preclinical studies in mice led to a significant reduction in human TAU, aggregation, and cell loss, resulting in a lifespan extension [134]. Testing of the gapmer ASO IONIS-MAPT<sub>Rx</sub> (NCT03186989) in non-human primates showed a *MAPT* mRNA reduction of nearly 80%. These promising preclinical experiments resulted in the initiation of a first clinical trial for patients with mild AD [123,135]. Further potential targets for AD treatment are other genes that are directly or indirectly related to Alzheimer's disease, such as *APP* and *APOE2*. The mRNA concentrations of these genes could also be reduced via steric block ASOs and may have beneficial effects on the course of AD [136,137].

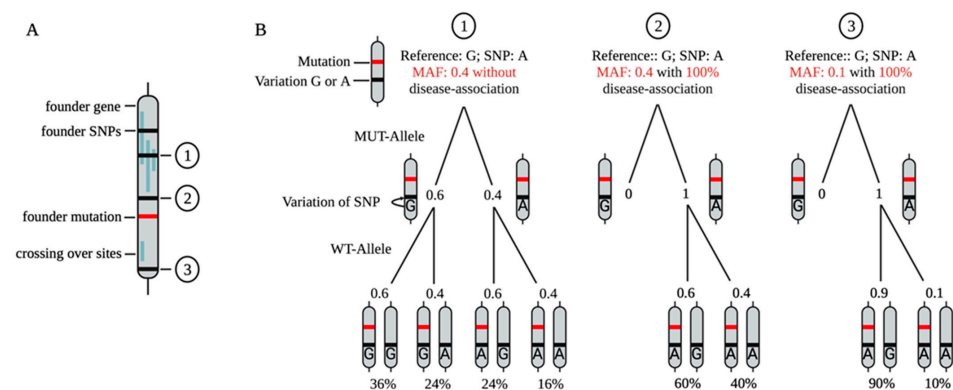
For Parkinson's disease, preclinical experiments in rodents achieved promising results in gapmer ASO treatment of different PD-associated genes [125,138,139]. Phase I and II clinical trials against both *LRRK2* and *SCNA* are ongoing (NCT03976349, NCT04165486).

## 7. SNP-Based Allele-Specific Treatment Strategies

Steric block ASOs that modulate splicing can be used to generate a semi-functional or non-functional protein. This shortened protein version lacks the toxic gain-of-function mutation of interest. Nevertheless, the total functional protein concentration is also lowered in this approach. Comparable effects can be achieved by non-allele-specific degradation via RNase H. Alternatively, an allele-specific degradation by targeting an SNP could surpass these non-allele-selective approaches. If the wild type allele and the disease-associated allele differ in one base at a specific site, this site might be a potential target for an allele-specific treatment strategy that aims to degrade the toxic allele at the (pre-)mRNA level. The association of a targetable SNP with the disease causing mutation might be ideal, but is not essential for such an approach. Even distant SNPs that are present in coding or non-coding regions of the disease gene far from the mutation can be used as a target to tag the disease-associated allele for degradation. The same principle can be used for steric block ASOs. Distant SNPs involved in splicing that are located in the same gene as the disease mutation could be used to disrupt the splicing machinery at the mutant allele.

In 2014, Skotte et al. published an approach to use HD-associated SNPs for allele-specific RNase H-mediated degradation of mHTT. They evaluated 50 previously published HD-associated SNPs for targetability [54,140,141]. A total of 4 SNPs were identified in which ASOs showed increased degradation. Further optimization of the ASO sequence increased selectivity from 2.4-fold to over 100-fold and reduced the IC<sub>50</sub> value against mHTT from over 400 nM to single digit nM values for an ASO targeting the SNP rs7685686\_A. To achieve this, the modifications of the flanking wings played an important role, as well as the shortening of the length of the ASO and the length of the DNA gap region. It was shown that a shorter gap, due to the shortened binding region of RNase H, resulted in enhanced

allele-selective degradation. Furthermore, this study demonstrated that the SNP does not need to be in the central position of the DNA gap to achieve allele-specific degradation. By targeting the selected SNP, it would be possible to treat 48.7% of the analyzed HD population allele-specifically and another 44.9% in a non-allele-specific manner [7]. In vivo follow-up studies of this ASO in humanized HD mice (Hu97/18) confirmed the allele-specific lowering of mtHTT and showed positive effects in behavioral tests [142,143]. In follow-up studies, Kay et al. precisely analyzed 63 SNPs in *HTT* from people of Canadian, Swedish, French, Italian, Korean, Japanese, Chinese, and European-Canadian ethnicity, revealing three major gene-spanning haplotype groups. Differences between controls and patients of each ethnic group were used to identify the most suitable SNPs for an allele-specific treatment approach, including the analysis of the association of some SNPs to the expanded CAG repeat [144,145]. In addition to SNPs, HD-associated insertion–deletion variations (indels) can potentially be used as targets for an allele-specific approach [146]. The identification and characterization of disease-associated SNPs is a crucial step towards the design of ASOs that can potentially be used to treat the majority of patients in an allele-specific manner (Figure 3).



**Figure 3.** Schematic description of the association between the mutation site and non-disease-causing SNPs. **(A)** Exemplary representation of a gene with its founder mutation (red) and individual additional variants/SNPs (black). Vertical blue shadings exemplarily indicate common crossover sites. The more crossovers occur within a region, the less the association between different regions of the gene, and the linkage disequilibrium (LD) decreases. **(B)** Numbers 1, 2, and 3 show potential SNPs in the founder gene with different association strengths to the mutation site after some generations. SNP 1 has a MAF of 0.4 and is located within a region with several common crossover sites. Therefore, the SNP shows no association to the founder mutation, even though the founder gene carries SNP 1. In a patient cohort, SNP 1 is therefore similarly distributed to a control cohort. A total of 60% of the patients carry a G in SNP 1 on the same allele as the founder mutation, and 40% carry an A. The WT allele of the patients show the same distribution in SNP 1. With an ASO that specifically targets SNP 1\_A, 24% of the cohort (A/G) could be treated as allele-specific, and an additional 16% (A/A) as non-allele-specific. SNP 2 has a MAF of 0.4 but is 100% associated with the disease mutation. Therefore, every patient carries an A in SNP 2, along with the mutation on the MUT allele. With an ASO that targets SNP 2\_A, 60% of the patients (A/G) could be treated as allele-specific and 40% as non-allele-specific. SNP 3 appears rarely in a population with an MAF of 0.1, but has an association of 100% to the mutation site. Every patient carries an A in SNP 3 on the same allele as the disease mutation. With an ASO that targets SNP 3\_A, 90% of the patients (A/G) can be treated as allele-specific, and the remaining 10% as non-allele-specific. These example pedigrees show the importance of long coding sequencing of the whole genes of patient cohorts. Targeting rare SNPs that have a high disease association might be an optimal target. Identification of these rare, disease-associated SNPs reduces the number of ASOs needed to identify suitable ASOs for an allele-specific approach in a whole patient cohort. MAF = minor allele frequency; SNP = single nucleotide polymorphism.

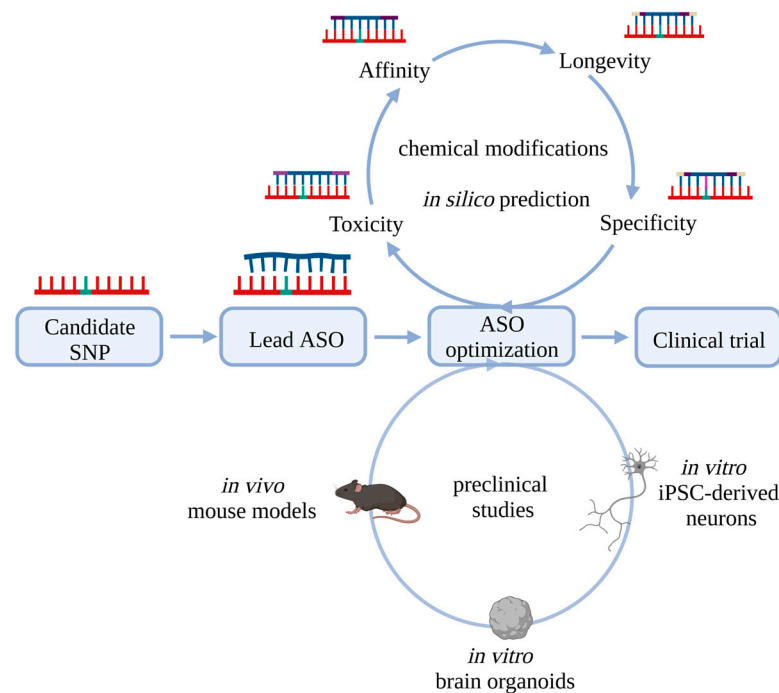
In a recent study, our group made use of an already known disease-associated SNP in *ATXN3* for an allele-specific ASO-based treatment in spinocerebellar ataxia type 3 (SCA3). Different ASOs were designed targeting a variant that is present in ~70% of SCA3 patients [6,147]. By establishing an in vitro platform using patient-derived iPSC-based cortical neurons, we were able to identify an ASO which leads to an allele-specific reduction in the glutamine-expanded allele by up to 75%. A one-time application showed significant allele-specific reduction for more than 7 weeks [6].

Together, these studies demonstrate that rare neurodegenerative diseases could play an important pioneering role in the development of allele-specific ASOs, especially when different modifications need to be tested in an in vitro screening. For this, an easily detectable and robust readout to quantify the allele-specific targeting efficiency is a crucial prerequisite. In the case of SCA3, iPSC-based patient-derived neuronal cultures are an ideal tool. Due to the repeat expansion, the mutant and wild type protein differ not only functionally, but also in their molecular mass. This difference can be used to display both forms separately at the protein level. Experience gained from an allele-specific design for ASOs targeting in HTT and SCA3 could then be transferred to diseases where allele-specific readout poses significant hurdles.

## 8. Target-Based ASO Design

One of the biggest challenges is to find a suitable target for an allele-specific ASO approach. If the variant that causes the disease can be directly targeted, it is usually well described. This becomes more difficult if new targets or SNPs need to be identified. As described, these can be SNPs that are located in the disease-causing gene, but are not causally related to the disease. To identify SNPs that are unrelated to the disease per se, but are in close linkage disequilibrium (LD) with a disease-associated mutation (point mutations or repeats), long-coding sequencing of larger cohorts would be of help (Figure 3). Alternatively, larger databases such as <https://www.ensembl.org/index.html> (accessed on 23 June 2022) or <https://bravo.sph.umich.edu/freeze8/hg38/> (accessed on 23 June 2022) can be used to identify a list of candidate SNPs. Suitable new targets should have a high MAF or a high association with the disease mutation (Figure 3). A hint could be given by the MAF or the linkage disequilibrium (LD) of the potential candidate SNP with the disease-causing variant (<https://ldlink.nci.nih.gov/?tab=home> (accessed on 23 June 2022)) [148]. Once a list of candidate SNPs has been generated, other factors can help with further selection. The target area of the (pre-)mRNA needs to be accessible for the ASO. Computations of potential 2D structures can predict easily or poorly accessible areas (<http://rna.tbi.univie.ac.at> (accessed on 23 June 2022)) [149]. Short sequence patterns within an ASO are expected to have positive or negative effects on ASO activity [150]. The probability of complementary off-targets can be calculated via GGGenome (<https://gggenome.dbcls.jp> (accessed on 23 June 2022)). Especially for an allele-specific approach, it is advantageous if the sequence is as short as possible. With each additional nucleotide, the affinity increases, while the contribution of the individual base to the total melting temperature decreases. Thus, the influence of a single match or mismatch decreases the longer an ASO is. In addition, base variation may also play a role. Some allelic SNP constellations might be more suitable for allele-specific targeting than others. Cytosines may be more suitable as mismatch partners, as they show a greater difference in melting temperature compared to the match [151,152]. In addition, neurotoxic effects of ASOs play an important role, especially for later application. Thus, the number and position of G's in an ASO sequence should be taken into account [70]. Once an appropriate SNP has been identified and the ASO designed, a suitable model is needed. For in vitro experiments, cells must be heterozygous for the targeted SNP and should ideally reflect the primarily affected organ/cell type. iPSC-derived cell cultures are particularly well suited for this purpose (Figure 4). They offer the advantage that they directly reflect a patient's heterozygous genotype and can be differentiated into any cell type of interest, including neuronal subtypes affected by the disease (reviewed in [153,154]). Therapeutic

effects and off-targets can easily be analyzed by transcriptomic and/or proteomic studies. (Neuro)toxicity can be quantified by, e.g., LDH assays or calcium oscillation measurements.



**Figure 4.** Workflow of in vitro testing and optimization of allele-specific ASOs. For allele-specific testing of ASOs, the association of the target with the mutation and the distribution in the population and patients should be known or determined. One or more lead ASOs could be tested at different positions, giving a first indication of the targetability of the (pre-)mRNA position. In further in vitro experiments, the lead ASO can be further modified to decrease toxicity and increase longevity, affinity, and allele-specificity. Different model systems have different advantages regarding evaluation of ASOs. iPSC-derived 2D models are a fast and cost-efficient tool to test the effectiveness of an ASO in a model with the genotype of a patient. Nevertheless, more complex systems, such as brain organoids, provide more precise information about the effects on the pathomechanism, such as aggregate formation, but also provide the opportunity to investigate off-targets in several neural cell types in parallel. Mice are still the model of choice to investigate (acute) toxicity and specificity in an in vivo system.

Another challenge is to find a suitable in vivo model, mainly mouse strains. This model should carry the target SNP on the same allele that bears the disease-associated variant. Lentiviral transduction of a humanized mutant allele with the respective target SNP has the advantage that the inserted allele and its SNPs can easily be adapted and that wild type mice can be used. Such transductions with the target allele can be performed prior to an ASO application [155,156]. In vivo mouse experiments are of particular importance for testing the in vivo functionality and acute toxicity of ASOs. Due to genomic differences between mice and humans, mouse models are less suitable for testing off-targets. Therefore, in vivo off-target studies need to be performed in non-human primates. Brain organoids from human iPSC may become increasingly important, especially in early in vitro off-target screenings (Figure 4). These self-assembled three-dimensional structures resemble the embryonic human brain and include multiple neural cell types, yielding the potential to extend the time of an ASO treatment in a multicellular system, which might be especially important for neurodegenerative diseases (reviewed in [157–159]).

## 9. The Challenge of Readout

In addition to the identification of appropriate SNPs, the identification of suitable read-outs to prove allele-specificity is another challenge that varies in difficulty between diseases. As previously mentioned, repeat expansion disorders, such as HD [7] or DRPLA [160], bear the advantage of different molecular weight between the wild type and expanded disease protein that can be used to discriminate the respective protein. In SCA3, the wild type and mutant allele typically differ by about 40 CAG repeats, resulting in an almost 6 kDa difference in molecular weight at the protein level. Thus, both proteins can easily be separated and discriminated by Western blot testing (WB) [6,161]. In other CAG repeat expansion diseases, such as SCA1, 2, or 6, absolute size differences are smaller, making it more challenging to separate the two proteins by WB. Longer runtimes and adjustment of gel type and gradient could help in solving this problem. Alternatively, proteins or mRNA could be predigested so that the difference in glutamine repeats relative to the total peptide becomes more distinct [103]. TR-FRET assays can also be used to discriminate between mutant and wild type protein. In this assay, a fluorophore coupled antibody binds specifically to the expanded polyQ part of the mutant protein, while a second antibody binds to a different part of the protein. Only when both antibodies bind does an energy transfer occur from one antibody to the other, resulting in a fluorescent signal that can be detected and quantified. A second pair of antibodies that binds both versions of the protein is used to determine total protein concentration [162].

A more elaborated approach would be to artificially create an *in vitro* model by transfecting cells with either the wild type sequence or a sequence with the corresponding SNP. Subsequently, cells could be treated with ASOs, which are then examined for their allele-specific effects. A fluorescence tag could help not only to control the transfection, but also to distinguish the transfected allele from the endogenous variant. Alternatively, cells could be transfected with both variants simultaneously, if the two variants have distinct tags. This would allow for studying allele-specific effects upon an ASO treatment directly in a cell culture model and later on, *in vivo*. A benefit of this approach could be that multiple SNP targets could be inserted into the mutant allele variant. Therefore, cooperative ASO effects could also be part of an *in vitro* investigation. With this approach, multiple ASOs initiate degradation of the same target by binding to different positions.

An indirect approach to test allele specificity of an ASO could be an RNase H assay. Both variants of the target RNA need to be incubated as a duplex with the corresponding ASO (with and without mismatch) and RNase H. In a fluorometric RNase H assay, the released DNA of the gapmer binds with a N-methyl mesoporphyrin IX (NMM), forming a G-quadruplex with a fluorescence signal [163], which can be amplified by using a catalytic hairpin assembly [164]. In a next step, the efficacy could be tested *in vitro* and *in vivo*.

## 10. Conclusions

Currently, no allele-specific ASO has been approved for the treatment of a neurodegenerative disease. However, an allele-selective approach with ASOs via RNase H degradation could have great potential, especially for toxic gain-of-function mutations. If successful, the toxic allele would be eliminated, and the wild type allele could continue to maintain its physiological function. In such an approach, all coding or non-coding SNPs that are on the same allele as the disease-causing variant are potential candidates for a personalized therapeutic approach [6,144,145], (Figure 3). One reason why current research is still in its early stages might be that the allele-specific readout for most diseases has major hurdles. Repeat expansion diseases, in which a readout might be established by a different molecular weight between the wild type and mutant protein, could be flagship projects for the development of allele-specific targeting therapies using ASOs.

To standardize the identification of targetable candidate SNPs for a specific disease, long-read sequencing can help (reviewed in [165]) to directly prove the disease-association of a candidate SNP. In case of a high association of a candidate SNP with the disease-causing mutation, these SNPs would be ideal starting points to design and test allele-specific ASOs,

including different modifications to increase allele-specificity. These ASOs could then be used to treat the majority of patients in an allele-specific manner.

Regarding ASO modifications, much time and effort has been spent on sugar phosphate backbone modifications. Since base modifications, such as G-clamp, can have the greatest impact on melting temperature per nucleotide [46,56] and therefore, the specificity and affinity of a single nucleotide, these modifications might be important to improve allele-selectivity. Another potential research focus for the future might be to analyze the cooperative effects of ASOs and whether there are ways to improve the administration of multiple different ASOs to enhance target engagement [166]. By administering a combination of ASOs, lower concentrations of each individual oligonucleotide could be used, reducing potential off-target effects of every single ASO. Thus, depending on the SNPs on the disease-associated allele, each patient could be given an ASO cocktail that specifically targets the mutant (pre)-mRNA for degradation through multiple binding sites.

Another hurdle is to establish a standardized workflow to identify and characterize ASOs in preclinical trials. We here propose a workflow for the design and preclinical testing of allele-specific ASOs (Figure 4). After successful target evaluation, several lead ASOs could be tested in parallel. The most effective of these will be further modified to ideally exclude toxicity and increase affinity, specificity, and longevity. Modified ASOs should then be characterized in vitro in 2D and 3D human cell culture models, as well as in vivo models. This includes effect-strength, allele-specificity, longevity, off-target effects, and (neuro)toxicity. While many challenges remain regarding allele-specific ASO therapies, allele-specific ASO strategies provide a great opportunity for future therapeutic approaches, especially for toxic gain-of-function neurodegenerative diseases.

**Author Contributions:** Conceptualization, J.H. and S.H.; writing—original draft preparation, J.H.; writing—review and editing, S.H. and L.S.; visualization, J.H. and S.H.; supervision, S.H. and L.S.; project administration, S.H. and L.S.; funding acquisition, S.H. and L.S. All authors have read and agreed to the published version of the manuscript.

**Funding:** The work of L.S. has been supported by the German Ministry of Education and Research (BMBF grant 01ED16028).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** Figures were created with [BioRender.com](https://www.biorender.com). We acknowledge support from the Open Access Publishing Fund of the University of Tübingen.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Testa, C.M. Antisense Oligonucleotide Therapeutics for Neurodegenerative Disorders. *Curr. Geriatr. Rep.* **2022**, *11*, 19–32. [[CrossRef](#)]
2. Rook, M.E.; Southwell, A.L. Antisense Oligonucleotide Therapy: From Design to the Huntington Disease Clinic. *BioDrugs* **2022**, *36*, 105–119. [[CrossRef](#)] [[PubMed](#)]
3. Roberts, T.C.; Langer, R.; Wood, M.J.A. Advances in oligonucleotide drug delivery. *Nat. Rev. Drug Discov.* **2020**, *19*, 673–694. [[CrossRef](#)] [[PubMed](#)]
4. Crooke, S.T.; Baker, B.F.; Crooke, R.M.; Liang, X.-H. Antisense technology: An overview and prospectus. *Nat. Rev. Drug Discov.* **2021**, *20*, 427–453. [[CrossRef](#)] [[PubMed](#)]
5. Scoles, D.R.; Minikel, E.V.; Pulst, S.M. Antisense oligonucleotides: A primer. *Neurol. Genet.* **2019**, *5*, e323. [[CrossRef](#)] [[PubMed](#)]
6. Hauser, S.; Helm, J.; Kraft, M.; Korneck, M.; Hübener-Schmid, J.; Schöls, L. Allele-specific targeting of mutant ataxin-3 by antisense oligonucleotides in SCA3-iPSC-derived neurons. *Mol. Ther.-Nucleic Acids* **2022**, *27*, 99–108. [[CrossRef](#)] [[PubMed](#)]
7. Skotte, N.H.; Southwell, A.L.; Østergaard, M.E.; Carroll, J.B.; Warby, S.C.; Doty, C.N.; Petoukhov, E.; Vaid, K.; Kordasiewicz, H.; Watt, A.T. Allele-specific suppression of mutant huntingtin using antisense oligonucleotides: Providing a therapeutic option for all Huntington disease patients. *PLoS ONE* **2014**, *9*, e107434. [[CrossRef](#)]
8. Taylor, J.P.; Hardy, J.; Fischbeck, K.H. Toxic Proteins in Neurodegenerative Disease. *Science* **2002**, *296*, 1991–1995. [[CrossRef](#)]

9. Scekcic-Zahirovic, J.; Sendscheid, O.; El Oussini, H.; Jambeau, M.; Sun, Y.; Mersmann, S.; Wagner, M.; Dieterlé, S.; Sinniger, J.; Dirrig-Grosch, S. Toxic gain of function from mutant FUS protein is crucial to trigger cell autonomous motor neuron loss. *EMBO J.* **2016**, *35*, 1077–1097. [\[CrossRef\]](#)
10. Yankner, B.A.; Lu, T. Amyloid  $\beta$ -protein toxicity and the pathogenesis of Alzheimer disease. *J. Biol. Chem.* **2009**, *284*, 4755–4759. [\[CrossRef\]](#)
11. Paulson, H.L.; Perez, M.; Trottier, Y.; Trojanowski, J.; Subramony, S.; Das, S.; Vig, P.; Mandel, J.-L.; Fischbeck, K.; Pittman, R. Intranuclear inclusions of expanded polyglutamine protein in spinocerebellar ataxia type 3. *Neuron* **1997**, *19*, 333–344. [\[CrossRef\]](#)
12. Ishikawa, K.; Nagai, Y. Molecular mechanisms and future therapeutics for spinocerebellar ataxia type 31 (SCA31). *Neurotherapeutics* **2019**, *16*, 1106–1114. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Chou, C.-C.; Zhang, Y.; Umoh, M.E.; Vaughan, S.W.; Lorenzini, I.; Liu, F.; Sayegh, M.; Donlin-Asp, P.G.; Chen, Y.H.; Duong, D.M. TDP-43 pathology disrupts nuclear pore complexes and nucleocytoplasmic transport in ALS/FTD. *Nat. Neurosci.* **2018**, *21*, 228–239. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Grima, J.C.; Daigle, J.G.; Arbez, N.; Cunningham, K.C.; Zhang, K.; Ochaba, J.; Geater, C.; Morozko, E.; Stocksdales, J.; Glatzer, J.C. Mutant huntingtin disrupts the nuclear pore complex. *Neuron* **2017**, *94*, 93–107.e6. [\[CrossRef\]](#) [\[PubMed\]](#)
15. MacDonald, M.E.; Novelletto, A.; Lin, C.; Tagle, D.; Barnes, G.; Bates, G.; Taylor, S.; Allitto, B.; Altherr, M.; Myers, R. The Huntington's disease candidate region exhibits many different haplotypes. *Nat. Genet.* **1992**, *1*, 99–103. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Simon-Sanchez, J.; Schulte, C.; Bras, J.M.; Sharma, M.; Gibbs, J.R.; Berg, D.; Paisan-Ruiz, C.; Lichtner, P.; Scholz, S.W.; Hernandez, D.G. Genome-wide association study reveals genetic risk underlying Parkinson's disease. *Nat. Genet.* **2009**, *41*, 1308–1312. [\[CrossRef\]](#)
17. Romanul, F.C.; Fowler, H.L.; Radvany, J.; Feldman, R.G.; Feingold, M. Azorean disease of the nervous system. *N. Engl. J. Med.* **1977**, *296*, 1505–1508. [\[CrossRef\]](#)
18. Morita, M.; Al-Chalabi, A.; Andersen, P.; Hosler, B.; Sapp, P.; Englund, E.; Mitchell, J.; Habgood, J.; De Belleruche, J.; Xi, J. A locus on chromosome 9p confers susceptibility to ALS and frontotemporal dementia. *Neurology* **2006**, *66*, 839–844. [\[CrossRef\]](#)
19. Chandran, J.S.; Scarrott, J.M.; Shaw, P.J.; Azzouz, M. Gene therapy in the nervous system: Failures and successes. *Pers. Med.* **2017**, *1007*, 241–257.
20. Seidel, K.; Schöls, L.; Nuber, S.; Petrasch-Parwez, E.; Gierga, K.; Wszolek, Z.; Dickson, D.; Gai, W.P.; Bornemann, A.; Riess, O. First appraisal of brain pathology owing to A30P mutant alpha-synuclein. *Ann. Neurol.* **2010**, *67*, 684–689. [\[CrossRef\]](#)
21. Metzger, S.; Rong, J.; Nguyen, H.-P.; Cape, A.; Tomiuk, J.; Soehn, A.S.; Propping, P.; Freudenberger-Hua, Y.; Freudenberger, J.; Tong, L.; et al. Huntingtin-associated protein-1 is a modifier of the age-at-onset of Huntington's disease. *Hum. Mol. Genet.* **2008**, *17*, 1137–1146. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Juliano, R.L. Chemical Manipulation of the Endosome Trafficking Machinery: Implications for Oligonucleotide Delivery. *Biomedicines* **2021**, *9*, 512. [\[CrossRef\]](#)
23. Crooke, S.T.; Wang, S.; Vickers, T.A.; Shen, W.; Liang, X.-h. Cellular uptake and trafficking of antisense oligonucleotides. *Nat. Biotechnol.* **2017**, *35*, 230–237. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Geary, R.S.; Norris, D.; Yu, R.; Bennett, C.F. Pharmacokinetics, biodistribution and cell uptake of antisense oligonucleotides. *Adv. Drug Deliv. Rev.* **2015**, *87*, 46–51. [\[CrossRef\]](#)
25. Wan, W.B.; Seth, P.P. The Medicinal Chemistry of Therapeutic Oligonucleotides. *J. Med. Chem.* **2016**, *59*, 9645–9667. [\[CrossRef\]](#)
26. Seth, P.P.; Siwkowski, A.; Allerson, C.R.; Vasquez, G.; Lee, S.; Prakash, T.P.; Wancewicz, E.V.; Witchell, D.; Swayze, E.E. Short Antisense Oligonucleotides with Novel 2'–4' Conformationally Restricted Nucleoside Analogues Show Improved Potency without Increased Toxicity in Animals. *J. Med. Chem.* **2009**, *52*, 10–13. [\[CrossRef\]](#)
27. Kurreck, J.; Wyszko, E.; Gillen, C.; Erdmann, V.A. Design of antisense oligonucleotides stabilized by locked nucleic acids. *Nucleic Acids Res.* **2002**, *30*, 1911–1918. [\[CrossRef\]](#) [\[PubMed\]](#)
28. Lu-Nguyen, N.; Malerba, A.; Herath, S.; Dickson, G.; Popplewell, L. Systemic antisense therapeutics inhibiting DUX4 expression ameliorates FSHD-like pathology in an FSHD mouse model. *Hum. Mol. Genet.* **2021**, *30*, 1398–1412. [\[CrossRef\]](#)
29. Synofzik, M.; van Roon-Mom, W.M.; Marckmann, G.; van Duyvenvoorde, H.A.; Graessner, H.; Schüle, R.; Aartsma-Rus, A. Preparing n-of-1 antisense oligonucleotide treatments for rare neurological diseases in Europe: Genetic, regulatory, and ethical perspectives. *Nucleic Acid Ther.* **2022**, *32*, 83–94. [\[CrossRef\]](#)
30. Crooke, S.T. Molecular mechanisms of antisense oligonucleotides. *Nucleic Acid Ther.* **2017**, *27*, 70–77. [\[CrossRef\]](#)
31. Lim, K.R.Q.; Yokota, T. Invention and early history of gapmers. *Gapmers* **2020**, *2176*, 3–19.
32. Lima, W.; Wu, H.; Crooke, S.T. The RNase H mechanism. *Antisense Drug Technol. Princ. Strateg. Appl.* **2007**, *2*, 47–74.
33. Liang, X.-H.; Sun, H.; Nichols, J.G.; Crooke, S.T. RNase H1-dependent antisense oligonucleotides are robustly active in directing RNA cleavage in both the cytoplasm and the nucleus. *Mol. Ther.* **2017**, *25*, 2075–2092. [\[CrossRef\]](#)
34. Freier, S.M.; Altmann, K.H. The ups and downs of nucleic acid duplex stability: Structure-stability studies on chemically-modified DNA:RNA duplexes. *Nucleic Acids Res.* **1997**, *25*, 4429–4443. [\[CrossRef\]](#)
35. Migawa, M.T.; Shen, W.; Wan, W.B.; Vasquez, G.; Oestergaard, M.E.; Low, A.; De Hoyos, C.L.; Gupta, R.; Murray, S.; Tanowitz, M.; et al. Site-specific replacement of phosphorothioate with alkyl phosphonate linkages enhances the therapeutic profile of gapmer ASOs by modulating interactions with cellular proteins. *Nucleic Acids Res.* **2019**, *47*, 5465–5479. [\[CrossRef\]](#) [\[PubMed\]](#)

36. Shen, W.; De Hoyos, C.L.; Migawa, M.T.; Vickers, T.A.; Sun, H.; Low, A.; Bell, T.A., 3rd; Rahdar, M.; Mukhopadhyay, S.; Hart, C.E.; et al. Chemical modification of PS-ASO therapeutics reduces cellular protein-binding and improves the therapeutic index. *Nat. Biotechnol.* **2019**, *37*, 640–650. [\[CrossRef\]](#)
37. Østergaard, M.E.; De Hoyos, C.L.; Wan, W.B.; Shen, W.; Low, A.; Berdeja, A.; Vasquez, G.; Murray, S.; Migawa, M.T.; Liang, X.-h.; et al. Understanding the effect of controlling phosphorothioate chirality in the DNA gap on the potency and safety of gapmer antisense oligonucleotides. *Nucleic Acids Res.* **2020**, *48*, 1691–1700. [\[CrossRef\]](#)
38. Stein, D.; Foster, E.; Huang, S.B.; Weller, D.; Summerton, J. A specificity comparison of four antisense types: Morpholino, 2'-O-methyl RNA, DNA, and phosphorothioate DNA. *Antisense Nucleic Acid Drug Dev.* **1997**, *7*, 151–157. [\[CrossRef\]](#)
39. Miroshnichenko, S.K.; Patutina, O.A.; Burakova, E.A.; Chelobanov, B.P.; Fokina, A.A.; Vlassov, V.V.; Altman, S.; Zenkova, M.A.; Stetsenko, D.A. Mesyl phosphoramidate antisense oligonucleotides as an alternative to phosphorothioates with improved biochemical and biological properties. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 1229–1234. [\[CrossRef\]](#)
40. Anderson, B.A.; Freestone, G.C.; Low, A.; De-Hoyos, C.L.; Iii, W.J.D.; Østergaard, M.E.; Migawa, M.T.; Fazio, M.; Wan, W.B.; Berdeja, A. Towards next generation antisense oligonucleotides: Mesylphosphoramidate modification improves therapeutic index and duration of effect of gapmer antisense oligonucleotides. *Nucleic Acids Res.* **2021**, *49*, 9026–9041. [\[CrossRef\]](#)
41. Lomzov, A.A.; Kupryushkin, M.S.; Shernyukov, A.V.; Nekrasov, M.D.; Dovydenko, I.S.; Stetsenko, D.A.; Pyshnyi, D.V. Diastereomers of a mono-substituted phosphoryl guanidine trideoxyribonucleotide: Isolation and properties. *Biochem. Biophys. Res. Commun.* **2019**, *513*, 807–811. [\[CrossRef\]](#) [\[PubMed\]](#)
42. Kupryushkin, M.S.; Filatov, A.V.; Mironova, N.L.; Patutina, O.A.; Chernikov, I.V.; Chernolovskaya, E.L.; Zenkova, M.A.; Pyshnyi, D.V.; Stetsenko, D.A.; Altman, S. Antisense oligonucleotide gapmers containing phosphoryl guanidine groups reverse MDR1-mediated multiple drug resistance of tumor cells. *Mol. Ther.-Nucleic Acids* **2022**, *27*, 211–226. [\[CrossRef\]](#) [\[PubMed\]](#)
43. Dyudeeva, E.; Kupryushkin, M.; Lomzov, A.; Pyshnaya, B.; Pyshnyi, D. Physicochemical properties of the phosphoryl guanidine oligodeoxyribonucleotide analogs. *Russ. J. Bioorg. Chem.* **2019**, *45*, 709–718. [\[CrossRef\]](#)
44. Skvortsova, Y.V.; Salina, E.G.; Burakova, E.A.; Bychenko, O.S.; Stetsenko, D.A.; Azhikina, T.L. A new antisense phosphoryl guanidine oligo-2'-O-methylribonucleotide penetrates into intracellular mycobacteria and suppresses target gene expression. *Front. Pharmacol.* **2019**, *10*, 1049. [\[CrossRef\]](#) [\[PubMed\]](#)
45. Bondensgaard, K.; Petersen, M.; Singh, S.K.; Rajwanshi, V.K.; Kumar, R.; Wengel, J.; Jacobsen, J.P. Structural studies of LNA: RNA duplexes by NMR: Conformations and implications for RNase H activity. *Chem.-Eur. J.* **2000**, *6*, 2687–2695. [\[CrossRef\]](#)
46. Stanton, R.; Sciabola, S.; Salatto, C.; Weng, Y.; Moshinsky, D.; Little, J.; Walters, E.; Kreeger, J.; DiMattia, D.; Chen, T. Chemical modification study of antisense gapmers. *Nucleic Acid Ther.* **2012**, *22*, 344–359. [\[CrossRef\]](#)
47. Singh, S.K.; Koshkin, A.A.; Wengel, J.; Nielsen, P. LNA (locked nucleic acids): Synthesis and high-affinity nucleic acid recognition. *Chem. Commun.* **1998**, 455–456. [\[CrossRef\]](#)
48. Koshkin, A.A.; Singh, S.K.; Nielsen, P.; Rajwanshi, V.K.; Kumar, R.; Meldgaard, M.; Olsen, C.E.; Wengel, J. LNA (Locked Nucleic Acids): Synthesis of the adenine, cytosine, guanine, 5-methylcytosine, thymine and uracil bicyclonucleoside monomers, oligomerisation, and unprecedented nucleic acid recognition. *Tetrahedron* **1998**, *54*, 3607–3630. [\[CrossRef\]](#)
49. Morita, K.; Hasegawa, C.; Kaneko, M.; Tsutsumi, S.; Sone, J.; Ishikawa, T.; Imanishi, T.; Koizumi, M. 2'-O, 4'-C-ethylene-bridged nucleic acids (ENA): Highly nuclease-resistant and thermodynamically stable oligonucleotides for antisense drug. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 73–76. [\[CrossRef\]](#)
50. Obika, S.; Nanbu, D.; Hari, Y.; Andoh, J.-i.; Morio, K.-i.; Doi, T.; Imanishi, T. Stability and structural features of the duplexes containing nucleoside analogues with a fixed N-type conformation, 2'-O, 4'-C-methylenribonucleosides. *Tetrahedron Lett.* **1998**, *39*, 5401–5404. [\[CrossRef\]](#)
51. Seth, P.P.; Vasquez, G.; Allerson, C.A.; Berdeja, A.; Gaus, H.; Kinberger, G.A.; Prakash, T.P.; Migawa, M.T.; Bhat, B.; Swayze, E.E. Synthesis and biophysical evaluation of 2', 4'-constrained 2' O-methoxyethyl and 2', 4'-constrained 2' O-ethyl nucleic acid analogues. *J. Org. Chem.* **2010**, *75*, 1569–1581. [\[CrossRef\]](#) [\[PubMed\]](#)
52. Egli, M.; Minasov, G.; Tereshko, V.; Pallan, P.S.; Teplova, M.; Inamati, G.B.; Lesnik, E.A.; Owens, S.R.; Ross, B.S.; Prakash, T.P.; et al. Probing the influence of stereoelectronic effects on the biophysical properties of oligonucleotides: Comprehensive analysis of the RNA affinity, nuclease resistance, and crystal structure of ten 2'-O-ribonucleic acid modifications. *Biochemistry* **2005**, *44*, 9045–9057. [\[CrossRef\]](#) [\[PubMed\]](#)
53. Teplova, M.; Minasov, G.; Tereshko, V.; Inamati, G.B.; Cook, P.D.; Manoharan, M.; Egli, M. Crystal structure and improved antisense properties of 2'-O-(2-methoxyethyl)-RNA. *Nat. Struct. Biol.* **1999**, *6*, 535–539. [\[CrossRef\]](#)
54. Østergaard, M.E.; Southwell, A.L.; Kordasiewicz, H.; Watt, A.T.; Skotte, N.H.; Doty, C.N.; Vaid, K.; Villanueva, E.B.; Swayze, E.E.; Frank Bennett, C. Rational design of antisense oligonucleotides targeting single nucleotide polymorphisms for potent and allele selective suppression of mutant Huntingtin in the CNS. *Nucleic Acids Res.* **2013**, *41*, 9634–9650. [\[CrossRef\]](#) [\[PubMed\]](#)
55. Seitz, O. Chemically Modified Antisense Oligonucleotides—Recent Improvements of RNA Binding and Ribonuclease H Recruitment. *Angew. Chem. Int. Ed.* **1999**, *38*, 3466–3469. [\[CrossRef\]](#)
56. Ortega, J.-A.; Blas, J.R.; Orozco, M.; Grandas, A.; Pedrosa, E.; Robles, J. Binding affinities of oligonucleotides and PNAs containing phenoxazine and G-clamp cytosine analogues are unusually sequence-dependent. *Org. Lett.* **2007**, *9*, 4503–4506. [\[CrossRef\]](#)
57. Froehler, B.C.; Wadwani, S.; Terhorst, T.J.; Gerrard, S.R. Oligodeoxynucleotides containing C-5 propyne analogs of 2'-deoxyuridine and 2'-deoxycytidine. *Tetrahedron Lett.* **1992**, *33*, 5307–5310. [\[CrossRef\]](#)

58. Herdewijn, P. Heterocyclic modifications of oligonucleotides and antisense technology. *Antisense Nucleic Acid Drug Dev.* **2000**, *10*, 297–310. [CrossRef]
59. Gutierrez, A.J.; Froehler, B.C. RNA duplex formation by oligodeoxynucleotides containing C-5 alkyne and C-5 thiazole substituted deoxyuridine analogs. *Tetrahedron Lett.* **1996**, *37*, 3959–3962. [CrossRef]
60. Østergaard, M.E.; Kumar, P.; Nichols, J.; Watt, A.; Sharma, P.K.; Nielsen, P.; Seth, P.P. Allele-selective inhibition of mutant Huntingtin with 2-thio-and C5-triazolylphenyl-deoxythymidine-modified antisense oligonucleotides. *Nucleic Acid Ther.* **2015**, *25*, 266–274. [CrossRef]
61. Dowdy, S.F. Overcoming cellular barriers for RNA therapeutics. *Nat. Biotechnol.* **2017**, *35*, 222–229. [CrossRef] [PubMed]
62. Crooke, S.T.; Vickers, T.A.; Liang, X.-h. Phosphorothioate modified oligonucleotide–protein interactions. *Nucleic Acids Res.* **2020**, *48*, 5235–5253. [CrossRef] [PubMed]
63. Putney, S.D.; Benkovic, S.J.; Schimmel, P.R. A DNA fragment with an alpha-phosphorothioate nucleotide at one end is asymmetrically blocked from digestion by exonuclease III and can be replicated in vivo. *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 7350–7354. [CrossRef] [PubMed]
64. Laurent, Q.; Martinent, R.; Moreau, D.; Winssinger, N.; Sakai, N.; Matile, S. Oligonucleotide Phosphorothioates Enter Cells by Thiol-Mediated Uptake. *Angew. Chem.* **2021**, *133*, 19250–19254. [CrossRef]
65. Eckstein, F. Phosphorothioate oligodeoxynucleotides: What is their origin and what is unique about them? *Antisense Nucleic Acid Drug Dev.* **2000**, *10*, 117–121. [CrossRef]
66. Iwamoto, N.; Butler, D.C.; Svrzikapa, N.; Mohapatra, S.; Zlatev, I.; Sah, D.W.; Standley, S.M.; Lu, G.; Apponi, L.H.; Frank-Kamenetsky, M. Control of phosphorothioate stereochemistry substantially increases the efficacy of antisense oligonucleotides. *Nat. Biotechnol.* **2017**, *35*, 845–851. [CrossRef]
67. Chelobanov, B.P.; Burakova, E.A.; Prokhorova, D.V.; Fokina, A.A.; Stetsenko, D.A. New oligodeoxynucleotide derivatives containing N-(methanesulfonyl)-phosphoramidate (mesyl phosphoramidate) internucleotide group. *Russ. J. Bioorganic Chem.* **2017**, *43*, 664–668. [CrossRef]
68. Prokhorova, D.; Chelobanov, B.; Burakova, E.; Fokina, A.; Stetsenko, D. New oligodeoxyribonucleotide derivatives bearing internucleotide N-tosyl phosphoramidate groups: Synthesis and complementary binding to DNA and RNA. *Russ. J. Bioorganic Chem.* **2017**, *43*, 38–42. [CrossRef]
69. Zhang, L.; Liang, X.-h.; De Hoyos, C.L.; Migawa, M.; Nichols, J.G.; Freestone, G.; Tian, J.; Seth, P.P.; Crooke, S.T. The Combination of Mesyl-Phosphoramidate Inter-Nucleotide Linkages and 2'-O-Methyl in Selected Positions in the Antisense Oligonucleotide Enhances the Performance of RNaseH1 Active PS-ASOs. *Nucleic Acid Ther.* **2022**. [CrossRef]
70. Hagedorn, P.H.; Brown, J.M.; Easton, A.; Pierdomenico, M.; Jones, K.; Olson, R.E.; Mercer, S.E.; Li, D.; Loy, J.; Høg, A.M.; et al. Acute Neurotoxicity of Antisense Oligonucleotides After Intracerebroventricular Injection Into Mouse Brain Can Be Predicted from Sequence Features. *Nucleic Acid Ther.* **2022**, *32*, 151–162. [CrossRef]
71. Patutina, O.A.; Gaponova, S.K.; Sen'kova, A.V.; Savin, I.A.; Gladkikh, D.V.; Burakova, E.A.; Fokina, A.A.; Maslov, M.A.; Shmendel', E.V.; Wood, M.J. Mesyl phosphoramidate backbone modified antisense oligonucleotides targeting miR-21 with enhanced in vivo therapeutic potency. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 32370–32379. [CrossRef] [PubMed]
72. Wave Life Science Provides Update on Phase 1b/2a PRECISION-HD Trials. Available online: <https://ir.wavelifesciences.com/node/9126/pdf> (accessed on 23 June 2022).
73. Liu, Y.; Dodart, J.-C.; Tran, H.; Berkovitch, S.; Braun, M.; Byrne, M.; Durbin, A.F.; Hu, X.S.; Iwamoto, N.; Jang, H.G. Variant-selective stereopure oligonucleotides protect against pathologies associated with C9orf72-repeat expansion in preclinical models. *Nat. Commun.* **2021**, *12*, 847. [CrossRef] [PubMed]
74. Kandasamy, P.; McClorey, G.; Shimizu, M.; Kothari, N.; Alam, R.; Iwamoto, N.; Kumarasamy, J.; Bommineni, G.R.; Bezigan, A.; Chivatakarn, O. Control of backbone chemistry and chirality boost oligonucleotide splice switching activity. *Nucleic Acids Res.* **2022**, *50*, 5443–5466. [CrossRef]
75. Brunet de Courssou, J.-B.; Durr, A.; Adams, D.; Corvol, J.-C.; Mariani, L.-L. Antisense therapies in neurological diseases. *Brain* **2022**, *145*, 816–831. [CrossRef]
76. Kupryushkin, M.; Pyshnyi, D.; Stetsenko, D. Phosphoryl guanidines: A new type of nucleic acid analogues. *Acta Nat.* **2014**, *6*, 116–118. [CrossRef]
77. Toulme, J.; Di Primo, C.; Moreau, S. Modulation of RNA function by oligonucleotides recognizing RNA structure. *Prog. Nucleic Acid Res. Mol. Biol.* **2001**, *69*, 1–46.
78. Lima, W.F.; Crooke, S.T. Binding affinity and specificity of Escherichia coli RNase H1: Impact on the kinetics of catalysis of antisense Oligonucleotide–RNA hybrids. *Biochemistry* **1997**, *36*, 390–398. [CrossRef] [PubMed]
79. Inoue, H.; Hayase, Y.; Iwai, S.; Ohtsuka, E. Sequence-dependent hydrolysis of RNA using modified oligonucleotide splints and RNase H. *FEBS Lett.* **1987**, *215*, 327–330. [CrossRef]
80. Burmeister, P.E.; Lewis, S.D.; Silva, R.F.; Preiss, J.R.; Horwitz, L.R.; Pendergrast, P.S.; McCauley, T.G.; Kurz, J.C.; Epstein, D.M.; Wilson, C.; et al. Direct in vitro selection of a 2'-O-methyl aptamer to VEGF. *Chem. Biol.* **2005**, *12*, 25–33. [CrossRef] [PubMed]
81. De Mesmaeker, A.; Haener, R.; Martin, P.; Moser, H.E. Antisense oligonucleotides. *Acc. Chem. Res.* **1995**, *28*, 366–374. [CrossRef]
82. Hoy, S.M. Nusinersen: First Global Approval. *Drugs* **2017**, *77*, 473–479. [CrossRef] [PubMed]
83. Khorkova, O.; Hsiao, J.; Wahlestedt, C. Nucleic Acid–Based Therapeutics in Orphan Neurological Disorders: Recent Developments. *Front. Mol. Biosci.* **2021**, *8*, 643681. [CrossRef] [PubMed]

84. Schobel, S.A. Preliminary results from GENERATION HD1, a phase III trial of tominersen in individuals with manifest HD. In Proceedings of the CHDI 16th Annual HD Therapeutics Conference, Online, 27–29 April 2021.
85. Obika, S.; Nanbu, D.; Hari, Y.; Morio, K.-i.; In, Y.; Ishida, T.; Imanishi, T. Synthesis of 2'-O, 4'-C-methyleuridine and-cytidine. Novel bicyclic nucleosides having a fixed C3'-endo sugar puckering. *Tetrahedron Lett.* **1997**, *38*, 8735–8738. [\[CrossRef\]](#)
86. Nielsen, K.E.; Rasmussen, J.; Kumar, R.; Wengel, J.; Jacobsen, J.P.; Petersen, M. NMR studies of fully modified locked nucleic acid (LNA) hybrids: Solution structure of an LNA: RNA hybrid and characterization of an LNA: DNA hybrid. *Bioconjug. Chem.* **2004**, *15*, 449–457. [\[CrossRef\]](#)
87. Swayze, E.E.; Siwkowski, A.M.; Wancewicz, E.V.; Migawa, M.T.; Wyrzykiewicz, T.K.; Hung, G.; Monia, B.P.; Bennett, C.F. Antisense oligonucleotides containing locked nucleic acid improve potency but cause significant hepatotoxicity in animals. *Nucleic Acids Res.* **2007**, *35*, 687–700. [\[CrossRef\]](#)
88. Seth, P.P.; Siwkowski, A.; Allerson, C.R.; Vasquez, G.; Lee, S.; Prakash, T.P.; Kinberger, G.; Migawa, M.T.; Gaus, H.; Bhat, B.; et al. Design, synthesis and evaluation of constrained methoxyethyl (cMOE) and constrained ethyl (cEt) nucleoside analogs. *Nucleic Acids Symp. Ser.* **2008**, 553–554. [\[CrossRef\]](#)
89. Krieg, A.M. Antiinfective applications of toll-like receptor 9 agonists. *Proc. Am. Thorac. Soc.* **2007**, *4*, 289–294. [\[CrossRef\]](#) [\[PubMed\]](#)
90. Varizhuk, A.M.; Zatsepin, T.S.; Golovin, A.V.; Belyaev, E.S.; Kostyukevich, Y.I.; Dedkov, V.G.; Shipulin, G.A.; Shpakovski, G.V.; Aralov, A.V. Synthesis of oligonucleotides containing novel G-clamp analogue with C8-tethered group in phenoxazine ring: Implication to qPCR detection of the low-copy Kemerovo virus dsRNA. *Bioorg. Med. Chem.* **2017**, *25*, 3597–3605. [\[CrossRef\]](#)
91. Egli, M.; Manoharan, M. Re-engineering RNA molecules into therapeutic agents. *Acc. Chem. Res.* **2019**, *52*, 1036–1047. [\[CrossRef\]](#)
92. Wilds, C.J.; Maier, M.A.; Tereshko, V.; Manoharan, M.; Egli, M. Direct observation of a cytosine analogue that forms five hydrogen bonds to guanosine: Guanidino G-clamp. *Angew. Chem. Int. Ed.* **2002**, *41*, 115–117. [\[CrossRef\]](#)
93. Tabrizi, S.J.; Leavitt, B.R.; Landwehrmeyer, G.B.; Wild, E.J.; Saft, C.; Barker, R.A.; Blair, N.F.; Craufurd, D.; Priller, J.; Rickards, H. Targeting huntingtin expression in patients with Huntington's disease. *N. Engl. J. Med.* **2019**, *380*, 2307–2316. [\[CrossRef\]](#) [\[PubMed\]](#)
94. Stoker, T.B.; Andresen, K.E.; Barker, R.A. Hydrocephalus complicating intrathecal antisense oligonucleotide therapy for Huntington's disease. *Mov. Disord.* **2021**, *36*, 263. [\[CrossRef\]](#) [\[PubMed\]](#)
95. Kwon, D. Failure of genetic therapies for Huntington's devastates community. *Nature* **2021**, *593*, 180. [\[CrossRef\]](#) [\[PubMed\]](#)
96. Arnold, C. Record number of gene-therapy trials, despite setbacks. *Nat. Med.* **2021**, *27*, 1312–1315. [\[CrossRef\]](#)
97. Bečanović, K.; Nørremølle, A.; Neal, S.J.; Kay, C.; Collins, J.A.; Arenillas, D.; Lilja, T.; Gaudenzi, G.; Manoharan, S.; Doty, C.N.; et al. A SNP in the HTT promoter alters NF-κB binding and is a bidirectional genetic modifier of Huntington disease. *Nat. Neurosci.* **2015**, *18*, 807–816. [\[CrossRef\]](#)
98. Moore, L.R.; Keller, L.; Bushart, D.D.; Delatorre, R.G.; Li, D.; McLoughlin, H.S.; do Carmo Costa, M.; Shakkottai, V.G.; Smith, G.D.; Paulson, H.L. Antisense oligonucleotide therapy rescues aggressive formation in a novel spinocerebellar ataxia type 3 human embryonic stem cell line. *Stem Cell Res.* **2019**, *39*, 101504. [\[CrossRef\]](#) [\[PubMed\]](#)
99. Moore, L.R.; Rajpal, G.; Dillingham, I.T.; Qutob, M.; Blumenstein, K.G.; Gattis, D.; Hung, G.; Kordasiewicz, H.B.; Paulson, H.L.; McLoughlin, H.S. Evaluation of antisense oligonucleotides targeting ATXN3 in SCA3 mouse models. *Mol. Ther.-Nucleic Acids* **2017**, *7*, 200–210. [\[CrossRef\]](#)
100. McLoughlin, H.S.; Moore, L.R.; Chopra, R.; Komlo, R.; McKenzie, M.; Blumenstein, K.G.; Zhao, H.; Kordasiewicz, H.B.; Shakkottai, V.G.; Paulson, H.L. Oligonucleotide therapy mitigates disease in spinocerebellar ataxia type 3 mice. *Ann. Neurol.* **2018**, *84*, 64–77. [\[CrossRef\]](#)
101. Winklhofer, K.F.; Tatzelt, J.; Haass, C. The two faces of protein misfolding: Gain-and loss-of-function in neurodegenerative diseases. *EMBO J.* **2008**, *27*, 336–349. [\[CrossRef\]](#)
102. Toonen, L.J.; Rigo, F.; van Attikum, H.; van Roon-Mom, W.M. Antisense oligonucleotide-mediated removal of the polyglutamine repeat in spinocerebellar ataxia type 3 mice. *Mol. Ther.-Nucleic Acids* **2017**, *8*, 232–242. [\[CrossRef\]](#)
103. Evers, M.M.; Pepers, B.A.; van Deutekom, J.C.; Mulders, S.A.; den Dunnen, J.T.; Aartsma-Rus, A.; van Ommen, G.-J.B.; van Roon-Mom, W.M. Targeting several CAG expansion diseases by a single antisense oligonucleotide. *PLoS ONE* **2011**, *6*, e24308. [\[CrossRef\]](#) [\[PubMed\]](#)
104. Hu, J.; Matsui, M.; Gagnon, K.T.; Schwartz, J.C.; Gabillet, S.; Arar, K.; Wu, J.; Bezprozvanny, I.; Corey, D.R. Allele-specific silencing of mutant huntingtin and ataxin-3 genes by targeting expanded CAG repeats in mRNAs. *Nat. Biotechnol.* **2009**, *27*, 478–484. [\[CrossRef\]](#) [\[PubMed\]](#)
105. Gagnon, K.T.; Pendergraff, H.M.; Deleavey, G.F.; Swayze, E.E.; Potier, P.; Randolph, J.; Roesch, E.B.; Chattopadhyaya, J.; Damha, M.J.; Bennett, C.F.; et al. Allele-Selective Inhibition of Mutant Huntingtin Expression with Antisense Oligonucleotides Targeting the Expanded CAG Repeat. *Biochemistry* **2010**, *49*, 10166–10178. [\[CrossRef\]](#)
106. Kourkouta, E.; Weij, R.; González-Barriga, A.; Mulder, M.; Verheul, R.; Bosgra, S.; Groenendaal, B.; Puoliväli, J.; Toivanen, J.; van Deutekom, J.C. Suppression of mutant protein expression in SCA3 and SCA1 mice using a CAG repeat-targeting antisense oligonucleotide. *Mol. Ther.-Nucleic Acids* **2019**, *17*, 601–614. [\[CrossRef\]](#)
107. Saudou, F.; Humbert, S. The biology of huntingtin. *Neuron* **2016**, *89*, 910–926. [\[CrossRef\]](#) [\[PubMed\]](#)
108. Freier, S.M. Compositions and Their Uses Directed to Huntingtin. U.S. Patent 8952145B2, 10 February 2015.
109. Brown, J.M.; Berkovitch, S.S.-M.; Iwamoto, N.; Vargeese, C.; Aklilu, K.M.; Frank-Kamenetsky, M.D.; Brown, D.P. Oligonucleotide Compositions and Methods Thereof. U.S. Patent 20220098585A1, 30 January 2020.

110. Nolan, M.; Talbot, K.; Ansorge, O. Pathogenesis of FUS-associated ALS and FTD: Insights from rodent models. *Acta Neuropathol. Commun.* **2016**, *4*, 99. [CrossRef]
111. Conte, A.; Lattante, S.; Zollino, M.; Marangi, G.; Luigetti, M.; Del Grande, A.; Servidei, S.; Trombetta, F.; Sabatelli, M. P525L FUS mutation is consistently associated with a severe form of juvenile amyotrophic lateral sclerosis. *Neuromuscul. Disord.* **2012**, *22*, 73–75. [CrossRef]
112. Zhang, W.; Jiao, B.; Xiao, T.; Liu, X.; Liao, X.; Xiao, X.; Guo, L.; Yuan, Z.; Yan, X.; Tang, B.; et al. Association of rare variants in neurodegenerative genes with familial Alzheimer's disease. *Ann. Clin. Transl. Neurol.* **2020**, *7*, 1985–1995. [CrossRef]
113. Korobeynikov, V.A.; Lyashchenko, A.K.; Blanco-Redondo, B.; Jafar-Nejad, P.; Shneider, N.A. Antisense oligonucleotide silencing of FUS expression as a therapeutic approach in amyotrophic lateral sclerosis. *Nat. Med.* **2022**, *28*, 104–116. [CrossRef]
114. McCord, J.M.; Fridovich, I. Superoxide dismutase: An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **1969**, *244*, 6049–6055. [CrossRef]
115. Miller, T.; Cudkowicz, M.; Shaw, P.J.; Andersen, P.M.; Atassi, N.; Bucelli, R.C.; Genge, A.; Glass, J.; Ladha, S.; Ludolph, A.L.; et al. Phase 1–2 Trial of Antisense Oligonucleotide Tofersen for SOD1 ALS. *N. Engl. J. Med.* **2020**, *383*, 109–119. [CrossRef] [PubMed]
116. Bennett, C.F.; Kenneth, W.D. Antisense Modulation of Superoxide Dismutase 1, Soluble Expression. U.S. Patent 8993529B2, 21 June 2011.
117. DeJesus-Hernandez, M.; Mackenzie, I.R.; Boeve, B.F.; Boxer, A.L.; Baker, M.; Rutherford, N.J.; Nicholson, A.M.; Finch, N.A.; Flynn, H.; Adamson, J. Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. *Neuron* **2011**, *72*, 245–256. [CrossRef] [PubMed]
118. Wave\_Life\_Sciences. Available online: <https://ir.wavelifesciences.com/static-files/4d0a51d3-35bc-471e-b5d8-b395ac5c7702> (accessed on 24 May 2022).
119. Liu, Y.; Andreucci, A.; Iwamoto, N.; Yin, Y.; Yang, H.; Liu, F.; Bulychiev, A.; Hu, X.S.; Lin, X.; Lamore, S. Preclinical evaluation of WVE-004, an investigational stereopure oligonucleotide for the treatment of C9orf72-associated ALS or FTD. *Mol. Ther.-Nucleic Acids* **2022**, *28*, 558–570. [CrossRef] [PubMed]
120. Albrecht, M.; Golatta, M.; Wüllner, U.; Lengauer, T. Structural and functional analysis of ataxin-2 and ataxin-3. *Eur. J. Biochem.* **2004**, *271*, 3155–3170. [CrossRef]
121. IONIS. Available online: <https://www.ionispharma.com/ionis-innovation/pipeline/> (accessed on 24 May 2022).
122. Rademakers, R.; Cruts, M.; Van Broeckhoven, C. The role of tau (MAPT) in frontotemporal dementia and related tauopathies. *Hum. Mutat.* **2004**, *24*, 277–295. [CrossRef]
123. Lane, R.M.; Kordasiewicz, H.B.; Smith, A.; Mignon, L.; Miller, T.; Narayanan, P.; Swayze, E.; Norris, D.; Fitzsimmons, B.; Bennett, C.F. Rationale for, and Development of, Ionis-MAPTRx, the First Tau-Lowering Antisense Oligonucleotide, in Patients with Mild AD. In Proceedings of the 142nd Annual Meeting of the American Neurological Association, San Diego, CA, USA, 15–17 October 2017; p. S151.
124. Usmani, A.; Shavarebi, F.; Hiniker, A. The Cell Biology of LRRK2 in Parkinson's Disease. *Mol. Cell. Biol.* **2021**, *41*, e00660-20. [CrossRef]
125. Zhao, H.T.; John, N.; Delic, V.; Ikeda-Lee, K.; Kim, A.; Weihofen, A.; Swayze, E.E.; Kordasiewicz, H.B.; West, A.B.; Volpicelli-Daley, L.A. LRRK2 antisense oligonucleotides ameliorate  $\alpha$ -synuclein inclusion formation in a Parkinson's disease mouse model. *Mol. Ther.-Nucleic Acids* **2017**, *8*, 508–519. [CrossRef]
126. Burré, J.; Sharma, M.; Tsetsenis, T.; Buchman, V.; Etherton, M.R.; Südhof, T.C.  $\alpha$ -Synuclein Promotes SNARE-Complex Assembly in Vivo and in Vitro. *Science* **2010**, *329*, 1663–1667. [CrossRef]
127. Alarcón-Arís, D.; Recasens, A.; Galofré, M.; Carballo-Carbajal, I.; Zacchi, N.; Ruiz-Bronchal, E.; Pavia-Collado, R.; Chica, R.; Ferrés-Coy, A.; Santos, M. Selective  $\alpha$ -synuclein knockdown in monoamine neurons by intranasal oligonucleotide delivery: Potential therapy for Parkinson's disease. *Mol. Ther.* **2018**, *26*, 550–567. [CrossRef]
128. Hagemann, T.L. Alexander disease: Models, mechanisms, and medicine. *Curr. Opin. Neurobiol.* **2022**, *72*, 140–147. [CrossRef] [PubMed]
129. Hagemann, T.L.; Powers, B.; Mazur, C.; Kim, A.; Wheeler, S.; Hung, G.; Swayze, E.; Messing, A. Antisense suppression of glial fibrillary acidic protein as a treatment for Alexander disease. *Ann. Neurol.* **2018**, *83*, 27–39. [CrossRef] [PubMed]
130. Smith, R.A.; Miller, T.M.; Yamanaka, K.; Monia, B.P.; Condon, T.P.; Hung, G.; Lobsiger, C.S.; Ward, C.M.; McAlonis-Downes, M.; Wei, H.; et al. Antisense oligonucleotide therapy for neurodegenerative disease. *J. Clin. Invest.* **2006**, *116*, 2290–2296. [CrossRef]
131. Donnelly, C.J.; Zhang, P.-W.; Pham, J.T.; Haeusler, A.R.; Mistry, N.A.; Vidensky, S.; Daley, E.L.; Poth, E.M.; Hoover, B.; Fines, D.M. RNA toxicity from the ALS/FTD C9ORF72 expansion is mitigated by antisense intervention. *Neuron* **2013**, *80*, 415–428. [CrossRef]
132. Tran, H.; Moazami, M.P.; Yang, H.; McKenna-Yasek, D.; Douthwright, C.L.; Pinto, C.; Metterville, J.; Shin, M.; Sanil, N.; Dooley, C. Suppression of mutant C9orf72 expression by a potent mixed backbone antisense oligonucleotide. *Nat. Med.* **2022**, *28*, 117–124. [CrossRef] [PubMed]
133. Miller, T.M.; Pestronk, A.; David, W.; Rothstein, J.; Simpson, E.; Appel, S.H.; Andres, P.L.; Mahoney, K.; Allred, P.; Alexander, K. An antisense oligonucleotide against SOD1 delivered intrathecally for patients with SOD1 familial amyotrophic lateral sclerosis: A phase 1, randomised, first-in-man study. *Lancet Neurol.* **2013**, *12*, 435–442. [CrossRef]
134. DeVos, S.L.; Miller, R.L.; Schoch, K.M.; Holmes, B.B.; Kebodeaux, C.S.; Wegener, A.J.; Chen, G.; Shen, T.; Tran, H.; Nichols, B.; et al. Tau reduction prevents neuronal loss and reverses pathological tau deposition and seeding in mice with tauopathy. *Sci. Transl. Med.* **2017**, *9*, eaag0481. [CrossRef]

135. Mignon, L.; Kordasiewicz, H.; Lane, R.; Smith, A.; Miller, T.; Narayanan, P.; Swayze, E.; Norris, D.; Fitzsimmons, B.; Bennett, F. Design of the first-in-human study of IONIS-MAPTRx, a tau-lowering antisense oligonucleotide, in patients with Alzheimer disease (S2. 006). *Neurology* **2018**, *90*, S2.006.
136. Chang, J.L.; Hinrich, A.J.; Roman, B.; Norrbom, M.; Rigo, F.; Marr, R.A.; Norstrom, E.M.; Hastings, M.L. Targeting amyloid- $\beta$  precursor protein, APP, splicing with antisense oligonucleotides reduces toxic amyloid- $\beta$  production. *Mol. Ther.* **2018**, *26*, 1539–1551. [\[CrossRef\]](#)
137. Hinrich, A.J.; Jodelka, F.M.; Chang, J.L.; Brutman, D.; Bruno, A.M.; Briggs, C.A.; James, B.D.; Stutzmann, G.E.; Bennett, D.A.; Miller, S.A.; et al. Therapeutic correction of ApoER2 splicing in Alzheimer's disease mice using antisense oligonucleotides. *EMBO Mol. Med.* **2016**, *8*, 328–345. [\[CrossRef\]](#)
138. Uehara, T.; Choong, C.-J.; Nakamori, M.; Hayakawa, H.; Nishiyama, K.; Kasahara, Y.; Baba, K.; Nagata, T.; Yokota, T.; Tsuda, H.; et al. Amido-bridged nucleic acid (AmNA)-modified antisense oligonucleotides targeting  $\alpha$ -synuclein as a novel therapy for Parkinson's disease. *Sci. Rep.* **2019**, *9*, 7567. [\[CrossRef\]](#) [\[PubMed\]](#)
139. Cole, T.A.; Zhao, H.; Collier, T.J.; Sandoval, I.; Sortwell, C.E.; Steece-Collier, K.; Daley, B.F.; Booms, A.; Lipton, J.; Welch, M.  $\alpha$ -Synuclein antisense oligonucleotides as a disease-modifying therapy for Parkinson's disease. *JCI Insight* **2021**, *6*, e135633. [\[CrossRef\]](#) [\[PubMed\]](#)
140. Carroll, J.B.; Warby, S.C.; Southwell, A.L.; Doty, C.N.; Greenlee, S.; Skotte, N.; Hung, G.; Bennett, C.F.; Freier, S.M.; Hayden, M.R. Potent and selective antisense oligonucleotides targeting single-nucleotide polymorphisms in the Huntington disease gene/allele-specific silencing of mutant huntingtin. *Mol. Ther.* **2011**, *19*, 2178–2185. [\[CrossRef\]](#) [\[PubMed\]](#)
141. Bennett, C.F.; Hayden, M.R.; Freier, S.M.; Greenlee, S.; Carroll, J.B.; Warby, S.C.; Swayze, E.E. Selective Reduction of Allelic Variants. U.S. Patent 20200377946, 14 January 2020.
142. Southwell, A.L.; Kordasiewicz, H.B.; Langbehn, D.R.; Skotte, N.H.; Parsons, M.P.; Villanueva, E.B.; Caron, N.S.; Østergaard, M.E.; Anderson, L.M.; Xie, Y.; et al. Huntingtin suppression restores cognitive function in a mouse model of Huntington's disease. *Sci. Transl. Med.* **2018**, *10*, eaar3959. [\[CrossRef\]](#) [\[PubMed\]](#)
143. Southwell, A.L.; Skotte, N.H.; Kordasiewicz, H.B.; Østergaard, M.E.; Watt, A.T.; Carroll, J.B.; Doty, C.N.; Villanueva, E.B.; Petoukhov, E.; Vaid, K. In vivo evaluation of candidate allele-specific mutant huntingtin gene silencing antisense oligonucleotides. *Mol. Ther.* **2014**, *22*, 2093–2106. [\[CrossRef\]](#)
144. Kay, C.; Collins, J.A.; Caron, N.S.; de Andrade Agostinho, L.; Findlay-Black, H.; Casal, L.; Sumathipala, D.; Dissanayake, V.H.; Cornejo-Olivas, M.; Baine, F. A comprehensive haplotype-targeting strategy for allele-specific HTT suppression in Huntington disease. *Am. J. Hum. Genet.* **2019**, *105*, 1112–1125. [\[CrossRef\]](#)
145. Kay, C.; Collins, J.A.; Skotte, N.H.; Southwell, A.L.; Warby, S.C.; Caron, N.S.; Doty, C.N.; Nguyen, B.; Griguoli, A.; Ross, C.J. Huntingtin haplotypes provide prioritized target panels for allele-specific silencing in Huntington disease patients of European ancestry. *Mol. Ther.* **2015**, *23*, 1759–1771. [\[CrossRef\]](#)
146. Shin, J.W.; Shin, A.; Park, S.S.; Lee, J.-M. Haplotype-specific insertion-deletion variations for allele-specific targeting in Huntington's disease. *Mol. Ther.-Methods Clin. Dev.* **2022**, *25*, 84–95. [\[CrossRef\]](#)
147. Gaspar, C.; Lopes-Cendes, I.; Hayes, S.; Goto, J.; Arvidsson, K.; Dias, A.; Silveira, I.; Maciel, P.; Coutinho, P.; Lima, M. Ancestral origins of the Machado-Joseph disease mutation: A worldwide haplotype study. *Am. J. Hum. Genet.* **2001**, *68*, 523–528. [\[CrossRef\]](#)
148. Machiela, M.J.; Chanock, S.J. LDlink: A web-based application for exploring population-specific haplotype structure and linking correlated alleles of possible functional variants. *Bioinformatics* **2015**, *31*, 3555–3557. [\[CrossRef\]](#)
149. Gruber, A.R.; Bernhart, S.H.; Lorenz, R. The ViennaRNA web services. In *RNA Bioinformatics*; Springer: Berlin/Heidelberg, Germany, 2015; pp. 307–326.
150. Matveeva, O.; Tsodikov, A.; Giddings, M.; Freier, S.; Wyatt, J.; Spiridonov, A.; Shabalina, S.; Gesteland, R.; Atkins, J. Identification of sequence motifs in oligonucleotides whose presence is correlated with antisense activity. *Nucleic Acids Res.* **2000**, *28*, 2862–2865. [\[CrossRef\]](#) [\[PubMed\]](#)
151. Wick, L.M.; Rouillard, J.M.; Whittam, T.S.; Gulari, E.; Tiedje, J.M.; Hashsham, S.A. On-chip non-equilibrium dissociation curves and dissociation rate constants as methods to assess specificity of oligonucleotide probes. *Nucleic Acids Res.* **2006**, *34*, e26. [\[CrossRef\]](#) [\[PubMed\]](#)
152. Naiser, T.; Ehler, O.; Kayser, J.; Mai, T.; Michel, W.; Ott, A. Impact of point-mutations on the hybridization affinity of surface-bound DNA/DNA and RNA/DNA oligonucleotide-duplexes: Comparison of single base mismatches and base bulges. *BMC Biotechnol.* **2008**, *8*, 48. [\[CrossRef\]](#) [\[PubMed\]](#)
153. Yousefi, N.; Abdollahii, S.; Kouhbanani, M.A.J.; Hassanzadeh, A. Induced pluripotent stem cells (iPSCs) as game-changing tools in the treatment of neurodegenerative disease: Mirage or reality? *J. Cell. Physiol.* **2020**, *235*, 9166–9184. [\[CrossRef\]](#)
154. Pasteuning-Vuhman, S.; de Jongh, R.; Timmers, A.; Pasterkamp, R.J. Towards advanced iPSC-based drug development for neurodegenerative disease. *Trends Mol. Med.* **2021**, *27*, 263–279. [\[CrossRef\]](#)
155. Gonçalves, N.; Simões, A.T.; Cunha, R.A.; de Almeida, L.P. Caffeine and adenosine A2A receptor inactivation decrease striatal neuropathology in a lentiviral-based model of Machado-Joseph disease. *Ann. Neurol.* **2013**, *73*, 655–666. [\[CrossRef\]](#)
156. Nobre, R.J.; Lobo, D.D.; Henriques, C.; Duarte, S.P.; Lopes, S.M.; Silva, A.C.; Lopes, M.M.; Mariet, F.; Schwarz, L.K.; Baatje, M. miRNA-Mediated Knockdown of ATXN3 Alleviates Molecular Disease Hallmarks in a Mouse Model for Spinocerebellar Ataxia Type 3. *Nucleic Acid Ther.* **2022**, *32*, 194–205. [\[CrossRef\]](#)

157. Grenier, K.; Kao, J.; Diamandis, P. Three-dimensional modeling of human neurodegeneration: Brain organoids coming of age. *Mol. Psychiatry* **2020**, *25*, 254–274. [[CrossRef](#)]
158. Slanzi, A.; Iannoto, G.; Rossi, B.; Zenaro, E.; Constantin, G. In vitro models of neurodegenerative diseases. *Front. Cell Dev. Biol.* **2020**, *8*, 328. [[CrossRef](#)]
159. Venkataraman, L.; Fair, S.R.; McElroy, C.A.; Hester, M.E.; Fu, H. Modeling neurodegenerative diseases with cerebral organoids and other three-dimensional culture systems: Focus on Alzheimer's disease. *Stem Cell Rev. Rep.* **2022**, *18*, 696–717. [[CrossRef](#)]
160. Hu, J.; Liu, J.; Narayanannair, K.J.; Lackey, J.G.; Kuchimanchi, S.; Rajeev, K.G.; Manoharan, M.; Swayze, E.E.; Lima, W.F.; Prakash, T.P. Allele-selective inhibition of mutant atrophin-1 expression by duplex and single-stranded RNAs. *Biochemistry* **2014**, *53*, 4510–4518. [[CrossRef](#)] [[PubMed](#)]
161. Hu, J.; Gagnon, K.T.; Liu, J.; Watts, J.K.; Syeda-Nawaz, J.; Bennett, C.F.; Swayze, E.E.; Randolph, J.; Chattopadhyaya, J.; Corey, D.R. Allele-selective inhibition of ataxin-3 (ATX3) expression by antisense oligomers and duplex RNAs. *Biol. Chem.* **2011**, *392*, 315–325. [[CrossRef](#)] [[PubMed](#)]
162. Gonsior, K.; Kaucher, G.A.; Pelz, P.; Schumann, D.; Gansel, M.; Kuhs, S.; Klockgether, T.; Forlani, S.; Durr, A.; Hauser, S. PolyQ-expanded ataxin-3 protein levels in peripheral blood mononuclear cells correlate with clinical parameters in SCA3: A pilot study. *J. Neurol.* **2021**, *268*, 1304–1315. [[CrossRef](#)] [[PubMed](#)]
163. Hu, D.; Pu, F.; Huang, Z.; Ren, J.; Qu, X. A Quadruplex-Based, Label-Free, and Real-Time Fluorescence Assay for RNase H Activity and Inhibition. *Chem.-Eur. J.* **2010**, *16*, 2605–2610. [[CrossRef](#)]
164. Lee, C.Y.; Jang, H.; Park, K.S.; Park, H.G. A label-free and enzyme-free signal amplification strategy for a sensitive RNase H activity assay. *Nanoscale* **2017**, *9*, 16149–16153. [[CrossRef](#)]
165. Kono, N.; Arakawa, K. Nanopore sequencing: Review of potential applications in functional genomics. *Dev. Growth Differ.* **2019**, *61*, 316–326. [[CrossRef](#)]
166. Echigoya, Y.; Lim, K.R.Q.; Melo, D.; Bao, B.; Trieu, N.; Mizobe, Y.; Maruyama, R.; Mamchaoui, K.; Tanihata, J.; Aoki, Y. Exons 45–55 skipping using mutation-tailored cocktails of antisense morpholinos in the DMD gene. *Mol. Ther.* **2019**, *27*, 2005–2017. [[CrossRef](#)]

# Exhibit 41

## HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use LEQVIO safely and effectively. See full prescribing information for LEQVIO.

**LEQVIO® (inclisiran) injection, for subcutaneous use**  
**Initial U.S. Approval: 2021**

### INDICATIONS AND USAGE

LEQVIO is a small interfering RNA (siRNA) directed to PCSK9 (proprotein convertase subtilisin kexin type 9) mRNA indicated as an adjunct to diet and maximally tolerated statin therapy for the treatment of adults with heterozygous familial hypercholesterolemia (HeFH) or clinical atherosclerotic cardiovascular disease (ASCVD), who require additional lowering of low-density lipoprotein cholesterol (LDL-C). (1)

#### Limitations of Use:

The effect of LEQVIO on cardiovascular morbidity and mortality has not been determined. (1)

### DOSAGE AND ADMINISTRATION

- The recommended dosage of LEQVIO, in combination with maximally tolerated statin therapy, is 284 mg administered as a single subcutaneous injection initially, again at 3 months, and then every 6 months. (2.1)

- LEQVIO should be administered by a healthcare professional. (2.2)
- Inject LEQVIO subcutaneously into the abdomen, upper arm, or thigh. (2.2)

### DOSAGE FORMS AND STRENGTHS

Injection: 284 mg/1.5 mL (189 mg/mL) in a single-dose prefilled syringe. (3)

### CONTRAINDICATIONS

None. (4)

### ADVERSE REACTIONS

Common adverse reactions in clinical trials ( $\geq 3\%$ ): injection site reaction, arthralgia, urinary tract infection, diarrhea, bronchitis, pain in extremity, and dyspnea. (6)

To report SUSPECTED ADVERSE REACTIONS, contact Novartis Pharmaceuticals Corporation at 1-888-669-6682 or FDA at 1-800-FDA-1088 or [www.fda.gov/medwatch](http://www.fda.gov/medwatch).

See 17 for PATIENT COUNSELING INFORMATION.

Revised: 12/2021

## FULL PRESCRIBING INFORMATION: CONTENTS\*

### 1 INDICATIONS AND USAGE

### 2 DOSAGE AND ADMINISTRATION

#### 2.1 Recommended Dosage

#### 2.2 Important Administration Instructions

### 3 DOSAGE FORMS AND STRENGTHS

### 4 CONTRAINDICATIONS

### 6 ADVERSE REACTIONS

#### 6.1 Clinical Trials Experience

#### 6.2 Immunogenicity

### 8 USE IN SPECIFIC POPULATIONS

#### 8.1 Pregnancy

#### 8.2 Lactation

#### 8.4 Pediatric Use

#### 8.5 Geriatric Use

#### 8.6 Renal Impairment

#### 8.7 Hepatic Impairment

### 11 DESCRIPTION

### 12 CLINICAL PHARMACOLOGY

#### 12.1 Mechanism of Action

#### 12.2 Pharmacodynamics

#### 12.3 Pharmacokinetics

### 13 NONCLINICAL TOXICOLOGY

#### 13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

### 14 CLINICAL STUDIES

### 16 HOW SUPPLIED/STORAGE AND HANDLING

### 17 PATIENT COUNSELING INFORMATION

\*Sections or subsections omitted from the full prescribing information are not listed.

## **FULL PRESCRIBING INFORMATION**

### **1 INDICATIONS AND USAGE**

LEQVIO® is indicated as an adjunct to diet and maximally tolerated statin therapy for the treatment of adults with heterozygous familial hypercholesterolemia (HeFH) or clinical atherosclerotic cardiovascular disease (ASCVD), who require additional lowering of low-density lipoprotein cholesterol (LDL-C).

#### Limitations of Use

The effect of LEQVIO on cardiovascular morbidity and mortality has not been determined.

### **2 DOSAGE AND ADMINISTRATION**

#### **2.1 Recommended Dosage**

The recommended dosage of LEQVIO, in combination with maximally tolerated statin therapy, is 284 mg administered as a single subcutaneous injection initially, again at 3 months, and then every 6 months.

If a planned dose is missed by less than 3 months, administer LEQVIO and maintain dosing according to the patient's original schedule.

If a planned dose is missed by more than 3 months, restart with a new dosing schedule - administer LEQVIO initially, again at 3 months, and then every 6 months.

Assess LDL-C when clinically indicated. The LDL-lowering effect of LEQVIO may be measured as early as 30 days after initiation and anytime thereafter without regard to timing of the dose.

#### **2.2 Important Administration Instructions**

LEQVIO should be administered by a healthcare professional.

Inject LEQVIO subcutaneously into the abdomen, upper arm, or thigh. Do not inject in areas of active skin disease or injury, such as sunburns, skin rashes, inflammation, or skin infections.

Inspect LEQVIO visually before use. It should appear clear and colorless to pale yellow. Do not use if particulate matter or discoloration is seen.

### **3 DOSAGE FORMS AND STRENGTHS**

Injection: 284 mg/1.5 mL (189 mg/mL) of inclisiran as a clear, and colorless to pale yellow solution in a single-dose prefilled syringe.

### **4 CONTRAINDICATIONS**

None.

### **6 ADVERSE REACTIONS**

#### **6.1 Clinical Trials Experience**

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

The data in Table 1 are derived from 3 placebo-controlled trials that included 1833 patients treated with LEQVIO, including 1682 exposed for 18 months (median treatment duration of 77 weeks) [see *Clinical Studies (14)*]. The mean age of the population was 64 years, 32% of the population were women, 92% were White, 6% were Black, 1% were Asian, and < 1% were other races. At baseline, 12% of patients had a diagnosis of heterozygous familial hypercholesterolemia and 85% had clinical atherosclerotic cardiovascular disease.

Adverse reactions reported in at least 3% of LEQVIO-treated patients, and more frequently than in placebo-treated patients, are shown in Table 1.

**Table 1: Adverse Reactions Occurring in Greater Than or Equal to 3% of LEQVIO-treated Patients and More Frequently than with Placebo (Studies 1, 2, and 3)**

Adverse Reactions	Placebo (N = 1822) %	LEQVIO (N = 1833) %
Injection site reaction†	1.8	8.2
Arthralgia	4.0	5.0
Urinary tract infection	3.6	4.4
Diarrhea	3.5	3.9
Bronchitis	2.7	4.3
Pain in extremity	2.6	3.3
Dyspnea	2.6	3.2

†includes related terms such as: injection site pain, erythema and rash

Adverse reactions led to discontinuation of treatment in 2.5% of patients treated with LEQVIO and 1.9% of patients treated with placebo. The most common adverse reactions leading to treatment discontinuation in patients treated with LEQVIO were injection site reactions (0.2% versus 0% for LEQVIO and placebo, respectively).

## 6.2 Immunogenicity

As with all oligonucleotides, there is potential for immunogenicity. The detection of antibody formation is highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of antibody (including neutralizing antibody) positivity in an assay may be influenced by several factors, including assay methodology, sample handling, timing of sample collection, concomitant medications, and underlying disease. For these reasons, comparison of the incidence of antibodies in the studies described below with the incidence of antibodies in other studies or to other products may be misleading.

The immunogenicity of LEQVIO has been evaluated using screening and confirmatory immunoassays for the detection of binding anti-drug antibodies to LEQVIO.

In the placebo-controlled clinical trials, 1830 patients had samples tested for anti-drug antibodies. Confirmed positivity was detected in 33 (1.8%) patients prior to dosing and in 90 (4.9%) patients during the 18 months of treatment with LEQVIO. Approximately 31 (1.7%) inclisiran-treated patients with a negative sample at baseline had a persistent anti-drug antibody response, defined as two confirmed positive samples separated by at least 16 weeks or a single confirmed positive final sample. There was no evidence that the presence of anti-drug binding antibodies impacted the pharmacodynamic profile, clinical response, or safety of LEQVIO, but the long-term consequences of continuing LEQVIO treatment in the presence of anti-drug binding antibodies are unknown.

## 8 USE IN SPECIFIC POPULATIONS

### 8.1 Pregnancy

#### Risk Summary

Discontinue LEQVIO when pregnancy is recognized. Alternatively, consider the ongoing therapeutic needs of the individual patient. Inclisiran increases LDL-C uptake and lowers LDL-C levels in the circulation, thus decreasing cholesterol and possibly other biologically active substances derived from cholesterol; therefore, LEQVIO may cause fetal harm when administered to pregnant patients based on the mechanism of action [*see Clinical Pharmacology (12.1)*]. In addition, treatment of hyperlipidemia is not generally necessary during pregnancy. Atherosclerosis is a chronic process and the discontinuation of lipid-lowering drugs during

pregnancy should have little impact on the outcome of long-term therapy of primary hyperlipidemia for most patients.

There are no available data on the use of LEQVIO in pregnant patients to evaluate for a drug-associated risk of major birth defects, miscarriage or adverse maternal or fetal outcomes.

In animal reproduction studies, no adverse developmental effects were observed in rats and rabbits with subcutaneous administration of inclisiran during organogenesis at doses up to 5 to 10 times the maximum recommended human dose (MRHD) based on body surface area (BSA) comparison (*see Data*). No adverse developmental outcomes were observed in offspring of rats administered inclisiran from organogenesis through lactation at 5 times the MRHD based on BSA comparison (*see Data*).

The estimated background risk of major birth defects and miscarriage for the indicated population is unknown. In the U.S. general population, the estimated background risk of major birth defects and miscarriage in clinically recognized pregnancies is 2%–4% and 15%–20%, respectively.

## Data

### *Animal Data*

In embryo-fetal development studies conducted in Sprague-Dawley rats and New Zealand White rabbits, inclisiran was administered by subcutaneous injection at dose levels of 50, 100, and 150 mg/kg once daily during organogenesis (rats: Gestation Days 6 to 17; rabbits: Gestation Days 7 to 19). There was no evidence of embryo-fetal toxicity or teratogenicity at doses up to 5 and 10 times, respectively, the MRHD based on BSA comparison/dose. Inclisiran crosses the placenta and was detected in rat fetal plasma at concentrations that were 65 to 154 times lower than maternal levels.

In a pre- and postnatal development study conducted in Sprague-Dawley rats, inclisiran was administered once daily by subcutaneous injection at levels of 50, 100, and 150 mg/kg from Gestation Day 6 through Lactation Day 20. Inclisiran was well-tolerated in maternal rats, with no evidence of maternal toxicity and no effects on maternal performance. There were no effects on the development of the F1 generation, including survival, growth, physical and reflexological development, behavior, and reproductive performance at doses up to 5 times the MRHD, based on BSA comparison/dose.

## **8.2 Lactation**

### Risk Summary

There is no information on the presence of inclisiran in human milk, the effects on the breastfed infant, or the effects on milk production. Inclisiran was present in the milk of lactating rats in all dose groups. When a drug is present in animal milk, it is likely that the drug will be present in human milk (*see Data*). Oligonucleotide-based products typically have poor oral bioavailability; therefore, it is considered unlikely that low levels of inclisiran present in milk will adversely impact an infant's development during lactation. The developmental and health benefits of breastfeeding should be considered along with the mother's clinical need for LEQVIO and any potential adverse effects on the breastfed infant from LEQVIO or from the underlying maternal condition.

## Data

In lactating rats, inclisiran was detected in milk at mean maternal plasma:milk ratios that ranged between 0.361 and 1.79. However, there is no evidence of systemic absorption in the suckling rat neonates.

## **8.4 Pediatric Use**

The safety and effectiveness of LEQVIO have not been established in pediatric patients.

## **8.5 Geriatric Use**

Of the 1833 patients treated with LEQVIO in clinical studies, 981 (54%) patients were 65 years of age and older, while 239 (13%) patients were 75 years of age and older. No overall differences in safety or effectiveness

were observed between these patients and younger patients, but greater sensitivity to adverse reactions of some older individuals cannot be ruled out.

## 8.6 Renal Impairment

No dose adjustments are necessary for patients with mild, moderate, or severe renal impairment [see *Clinical Pharmacology* (12.3)]. LEQVIO has not been studied in patients with end stage renal disease [see *Clinical Pharmacology* (12.3)].

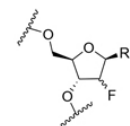
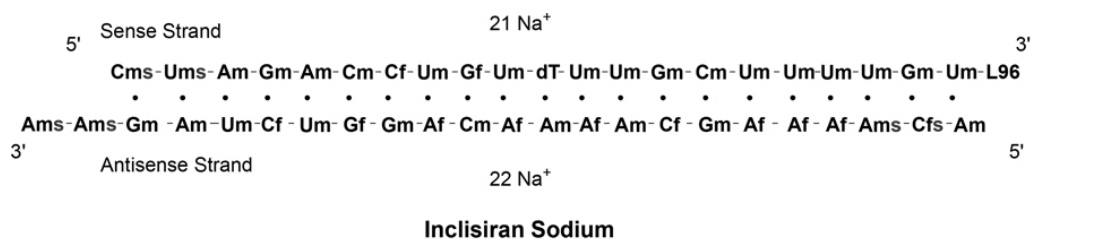
## 8.7 Hepatic Impairment

No dose adjustment is necessary in patients with mild to moderate hepatic impairment. LEQVIO has not been studied in patients with severe hepatic impairment [see *Clinical Pharmacology* (12.3)].

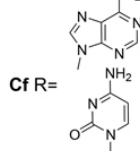
## 11 DESCRIPTION

LEQVIO contains inclisiran sodium, a small interfering RNA (siRNA) directed to PCSK9 (proprotein convertase subtilisin kexin type 9) mRNA. Inclisiran contains a covalently linked ligand containing three N-acetylgalactosamine (GalNAc) residues to facilitate delivery to hepatocytes. With one exception, the 2'ribose moieties of the inclisiran sodium are present as 2'-F or 2'-OMe ribonucleotide. In addition, six of the terminal phosphodiester backbones are present as phosphorothioate linkages as indicated below.

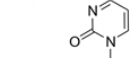
The molecular formula of inclisiran sodium is  $C_{529}H_{664}F_{12}N_{176}Na_{43}O_{316}P_{43}S_6$  and its molecular weight is 17,284.72 g/mol. It has the following structural formula:



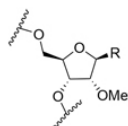
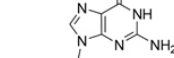
**2'-F nucleotides**  
Af R =



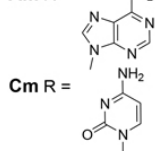
Cf R =



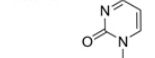
Gf R =



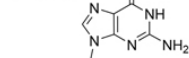
**2'-OMe nucleotides**  
Am R =



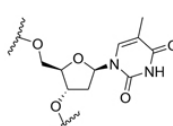
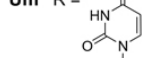
Cm R =



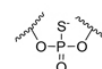
Gm R =



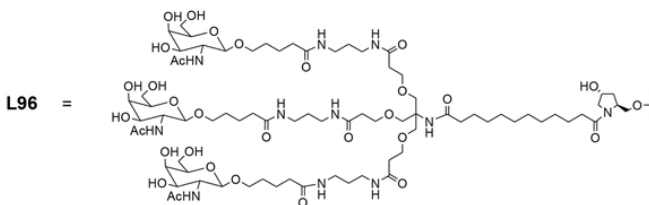
Um R =



dT = thymidine



s = phosphorothioate



Abbreviations: Af = adenine 2'-F ribonucleotide; Cf = cytosine 2'-F ribonucleotide; Gf = guanine 2'-F ribonucleotide; Am = adenine 2'-OMe ribonucleotide; Cm = cytosine 2'-OMe ribonucleotide; Gm = guanine 2'-OMe ribonucleotide; Um = uracil 2'-OMe ribonucleotide; L96 = triantennary GalNAc (N-acetyl-galactosamine)

LEQVIO is a sterile, preservative-free, clear, and colorless to pale yellow solution for subcutaneous use in a prefilled syringe. Each syringe contains 1.5 mL of solution containing the equivalent of 284 mg inclisiran (present as 300 mg inclisiran sodium salt). LEQVIO is formulated in Water for Injection and may also contain sodium hydroxide and/or phosphoric acid for pH adjustment to a target pH of 7.0.

## 12 CLINICAL PHARMACOLOGY

### 12.1 Mechanism of Action

Inclisiran is a double-stranded small interfering ribonucleic acid (siRNA), conjugated on the sense strand with triantennary N-Acetylgalactosamine (GalNAc) to facilitate uptake by hepatocytes. In hepatocytes, inclisiran utilizes the RNA interference mechanism and directs catalytic breakdown of mRNA for PCSK9. This increases LDL-C receptor recycling and expression on the hepatocyte cell surface, which increases LDL-C uptake and lowers LDL-C levels in the circulation.

### 12.2 Pharmacodynamics

Following a single subcutaneous administration of 284 mg of inclisiran, LDL-C reduction was apparent within 14 days post dose. Mean reductions of 38% to 51% for LDL-C were observed 30 to 180 days post dose. At Day 180, LDL-C levels were still reduced by approximately 53%.

Following a dose at Day 1 and Day 90 of 284 mg of inclisiran, mean serum PCSK9 levels were reduced by approximately 75% and 69% at Day 120, and Day 180, respectively.

In the clinical studies, following four doses of LEQVIO at Day 1, Day 90 (3 months), Day 270 (~6 months) and Day 450 (~12 months), LDL-C, total cholesterol, ApoB, and non-HDL-C were reduced [*see Clinical Studies (14)*].

#### Cardiac Electrophysiology

At a dose 3 times the maximum recommended dose, inclisiran does not prolong the QT interval to any clinically relevant extent.

### 12.3 Pharmacokinetics

#### Absorption

Following a single subcutaneous administration, systemic exposure to inclisiran increased in a linear and dose proportional manner over a range from 25 mg to 800 mg of inclisiran sodium. At the recommended dosing regimen of 284 mg of LEQVIO, plasma concentrations reached peak in approximately 4 hours post dose with a mean  $C_{max}$  of 509 ng/mL. Concentrations reached undetectable levels after 24 to 48 hours post dosing. The mean area under the plasma concentration-time curve from dosing extrapolated to infinity was 7980 ng\*h/mL. Pharmacokinetic findings following multiple subcutaneous administrations of LEQVIO were similar to single-dose administration.

#### Distribution

Inclisiran is 87% protein bound *in vitro* at the relevant clinical plasma concentrations. Following a single subcutaneous 284 mg dose of LEQVIO to healthy adults, the apparent volume of distribution is approximately 500 L. Inclisiran has been shown to have high uptake into, and selectively for the liver, the target organ for cholesterol lowering.

#### Elimination

The terminal elimination half-life of LEQVIO is approximately 9 hours, and no accumulation occurs with multiple dosing. Approximately 16% of LEQVIO is cleared through the kidney.

#### Metabolism

Inclisiran is primarily metabolized by nucleases to shorter nucleotides of varying length. Inclisiran is not a substrate for CYP450 or transporters.

#### Specific Populations

A population pharmacodynamic analysis was conducted on data from 4328 patients. Age, body weight, gender, race, and creatinine clearance were found not to significantly influence inclisiran pharmacokinetics.

### *Renal Impairment*

Pharmacokinetic analysis of data from a dedicated renal impairment study reported increases in inclisiran  $C_{max}$  and AUC of approximately 2.3 to 3.3-fold and 1.6 to 2.3-fold, respectively, in patients with mild, moderate or severe renal impairment, relative to patients with normal renal function. Despite the higher plasma exposures, reductions in LDL-C were similar across all groups based on renal function.

### *Hepatic Impairment*

Pharmacokinetic analysis of data from a dedicated hepatic impairment study reported increases in inclisiran  $C_{max}$  and AUC of approximately 1.1- to 2.1-fold and 1.3- to 2.0-fold, respectively, in patients with mild and moderate hepatic impairment, relative to patients with normal hepatic function. Despite the higher plasma inclisiran exposures, reductions in LDL-C were similar between the groups of patients administered inclisiran with normal hepatic function and mild hepatic impairment. In patients with moderate hepatic impairment, baseline PCSK9 levels were lower and reductions in LDL-C were less than those observed in patients with normal hepatic function. LEQVIO has not been studied in patients with severe hepatic impairment.

### Drug Interaction Studies

No formal clinical drug interaction studies have been performed. The components of LEQVIO are not substrates, inhibitors or inducers of cytochrome P450 enzymes or transporters. In a population pharmacokinetic analysis, concomitant use of inclisiran did not have a clinically significant impact on atorvastatin or rosuvastatin concentrations. LEQVIO is not expected to cause drug-drug interactions or to be affected by inhibitors or inducers of cytochrome P450 enzymes or transporters.

## **13 NONCLINICAL TOXICOLOGY**

### **13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility**

In a 2-year carcinogenicity study, Sprague-Dawley rats were administered subcutaneous doses of 40, 95, or 250 mg/kg inclisiran once every 28 days (1, 3, or 8 times the MRHD, based on BSA comparison/dose). Inclisiran was not carcinogenic up to the highest dose tested.

In a 26-week study in RasH2Tg mice, subcutaneous doses of 300, 600, or 1,500 mg/kg once every 28 days were administered. Inclisiran was not carcinogenic up to the highest dose tested.

Inclisiran was not mutagenic or clastogenic in a standard battery of genotoxicity tests, including a bacterial mutagenicity assay, an *in vitro* chromosome aberration assay using human peripheral lymphocytes, and an *in vivo* bone marrow micronucleus assay in rats.

Fertility and early embryonic-development studies were conducted in male and female rats. In male rats, inclisiran was administered subcutaneously at dose levels of 10, 50, and 250 mg/kg every 2 weeks for 4 weeks before cohabitation through mating, and until termination between Days 64 and 67. In female rats, inclisiran was administered subcutaneously at dose levels 10, 50, and 250 mg/kg once every 4 days beginning 14 days prior to cohabitation and through mating, followed by 10, 50, or 150 mg/kg once daily during the gestation period up to Gestation Day 7. There were no adverse effects on fertility up to the highest dose examined, corresponding to 8 times the MRHD, based on BSA comparison/dose.

## **14 CLINICAL STUDIES**

The efficacy of LEQVIO was investigated in three randomized, double-blind, placebo-controlled trials that enrolled 3457 adults with HeFH or clinical ASCVD, who were taking maximally tolerated statin therapy and who required additional LDL-C lowering. Demographics and baseline disease characteristics were balanced between the treatment arms in all trials.

LDL-C Reduction in Patients with Clinical Atherosclerotic Cardiovascular Disease

Study 1 (ORION-10, NCT03399370) was a multicenter, double-blind, randomized, placebo-controlled 18-month trial in which 1561 patients with ASCVD were randomized 1:1 to receive subcutaneous injections of either LEQVIO 284 mg (n = 781) or placebo (n = 780) on Day 1, Day 90, Day 270, and at Day 450. Patients were taking a maximally tolerated dose of statin with or without other lipid modifying therapy, and required additional LDL-C reduction. Patients were stratified by current use of statins or other lipid-modifying therapies. Patients taking PCSK9 inhibitors were excluded from the trial.

The mean age at baseline was 66 years (range: 35 to 90 years), 60% were  $\geq 65$  years old, 31% were women, 86% were White, 13% were Black, 1% were Asian and 14% identified as Hispanic or Latino ethnicity. Forty-five percent (45%) of patients had diabetes at baseline. The mean baseline LDL-C was 105 mg/dL. At the time of randomization, 89% of patients were receiving statin therapy and 69% were receiving high-intensity statin therapy.

The primary efficacy outcome measure in Study 1 was the percent change from baseline to Day 510 in LDL-C. The difference between the LEQVIO and placebo groups in mean percentage change in LDL-C from baseline to Day 510 was -52% (95% CI: -56%, -49%;  $p < 0.0001$ ). For additional results, see Table 2 and Figure 1.

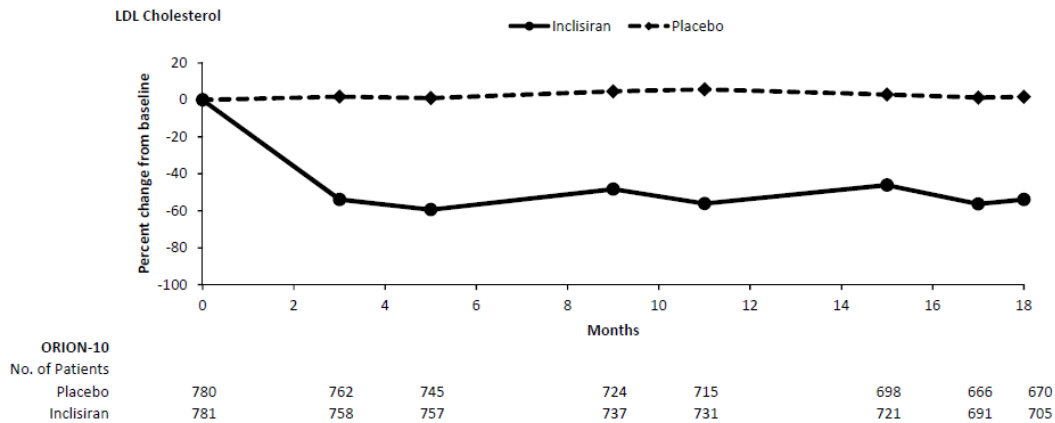
**Table 2: Changes in Lipid Parameters in Patients with ASCVD on Maximally Tolerated Statin Therapy (Mean % Change from Baseline to Day 510 in Study 1)**

Treatment Group	LDL-C	Total Cholesterol	Non-HDL-C	ApoB
Day 510 (mean percentage change from baseline)				
Placebo (n = 780)	1	0	0	-2
LEQVIO (n = 781)	-51	-34	-47	-45
Difference from placebo (LS Mean) (95% CI)	-52 (-56, -49)	-33 (-35, -31)	-47 (-50, -44)	-43 (-46, -41)

ApoB = apolipoprotein B; CI = confidence interval; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol

11.5% of subjects on LEQVIO and 14.6% of subjects on placebo had missing LDL-C data at primary endpoint (Day 510). Missing data were imputed using a modified control-based multiple imputation to account for treatment adherence. Percent change from baseline in LDL-C was analyzed using analysis of covariance (ANCOVA) with fixed effect for treatment group and baseline LDL-C as a covariate. Other endpoints were analyzed using a mixed-effect model for repeated measure (MMRM) with fixed effects for treatment group, visit, interaction between treatment and visit, and baseline value. Missing data were imputed using a control-based pattern-mixture model approach.

**Figure 1: Mean Percent Change from Baseline in LDL-C Over 18 Months in Patients with ASCVD on Maximally Tolerated Statin Therapy (Study 1)**



Study 2 (ORION-11, NCT03400800) was a multicenter, double-blind, randomized, placebo-controlled 18-month trial in which 1414 adults with ASCVD were randomized 1:1 to receive subcutaneous injections of either LEQVIO 284 mg (n = 712) or placebo (n = 702) on Day 1, Day 90, Day 270, and Day 450. Patients were taking a maximally tolerated dose of statin with or without other lipid modifying therapy, and required additional LDL-C reduction. Patients were stratified by country and by current use of statins or other lipid-modifying therapies. Patients taking PCSK9 inhibitors were excluded from the trial.

The mean age at baseline was 65 years (range: 35 to 88 years), 56% were  $\geq 65$  years old, 25% were women, 98% were White, 1% were Black, < 1% were Asian, and 1% identified as Hispanic or Latino ethnicity. Thirty-one percent (31%) of patients had diabetes at baseline. The mean baseline LDL-C was 101 mg/dL. At the time of randomization, 96% of patients were receiving statin therapy and 80% were receiving high-intensity statin therapy.

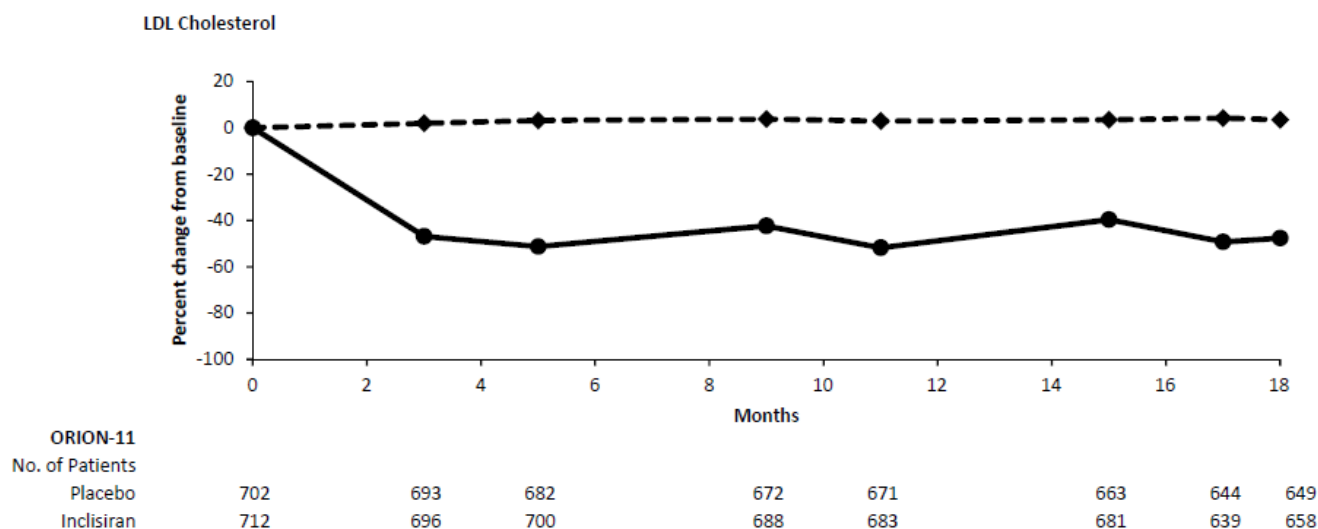
The primary efficacy outcome measure in Study 2 was the percent change from baseline to Day 510 in LDL-C. The difference between the LEQVIO and placebo groups in mean percentage change in LDL-C from baseline to Day 510 was -51% (95% CI: -54%, -47%;  $p < 0.0001$ ). For additional results, see Table 3 and Figure 2.

**Table 3: Changes in Lipid Parameters in Patients with ASCVD on Maximally Tolerated Statin Therapy (Mean % Change from Baseline to Day 510 in Study 2)**

Treatment Group	LDL-C	Total Cholesterol	Non-HDL-C	ApoB
Day 510 (mean percentage change from baseline)				
Placebo (n = 702)	4	2	2	1
LEQVIO (n = 712)	-46	-28	-42	-39
Difference from placebo (LS Mean) (95% CI)	-51 (-54, -47)	-30 (-32, -28)	-44 (-47, -41)	-40 (-42, -37)

ApoB = apolipoprotein B; CI = confidence interval; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol

10.3% of subjects on LEQVIO and 8.3% of subjects on placebo had missing LDL-C data at primary endpoint (Day 510). Missing data were imputed using a modified control-based multiple imputation to account for treatment adherence. Percent change from baseline in LDL-C was analyzed using analysis of covariance (ANCOVA) with fixed effect for treatment group and baseline LDL-C as a covariate. Other endpoints were analyzed using mixed-effect model for repeated measure (MMRM) with fixed effects for treatment group, visit, interaction between treatment and visit, and baseline value. Missing data were imputed using a control-based pattern-mixture model approach.

**Figure 2: Mean Percent Change from Baseline in LDL-C Over 18 Months in Patients with ASCVD on Maximally Tolerated Statin Therapy (Study 2)**

In a pooled analysis of Study 1 and Study 2, the observed treatment effect was similar across predefined subgroups, such as sex, age, race, disease characteristics, geographic regions, presence of diabetes, body mass index, baseline LDL-C levels, and intensity of statin treatment.

#### Heterozygous Familial Hypercholesterolemia (HeFH)

Study 3 (ORION-9, NCT03397121) was a multicenter, double-blind, randomized, placebo-controlled 18-month trial in which 482 patients with HeFH were randomized 1:1 to receive subcutaneous injections of either LEQVIO 284 mg (n = 242) or placebo (n = 240) on Day 1, Day 90, Day 270, and at Day 450. Patients with HeFH were taking a maximally tolerated dose of statin with or without other lipid modifying therapy, and required additional LDL-C reduction. The diagnosis of HeFH was made either by genotyping or clinical criteria using either the Simon Broome or WHO/Dutch Lipid Network criteria. Patients were stratified by country and by current use of statins or other lipid-modifying therapies. Patients taking PCSK9 inhibitors were excluded from the trial.

The mean age at baseline was 55 years (range: 21 to 80 years), 22% were  $\geq 65$  years old, 53% were women, 94% were White, 3% were Black, 3% were Asian and 3% identified as Hispanic or Latino ethnicity. Ten percent (10%) of patients had diabetes at baseline. The mean baseline LDL-C was 153 mg/dL. At the time of randomization, 90% of patients were receiving statin therapy and 74% were receiving high-intensity statin therapy. Fifty-two percent (52%) of patients were treated with ezetimibe. The most commonly administered statins were atorvastatin and rosuvastatin.

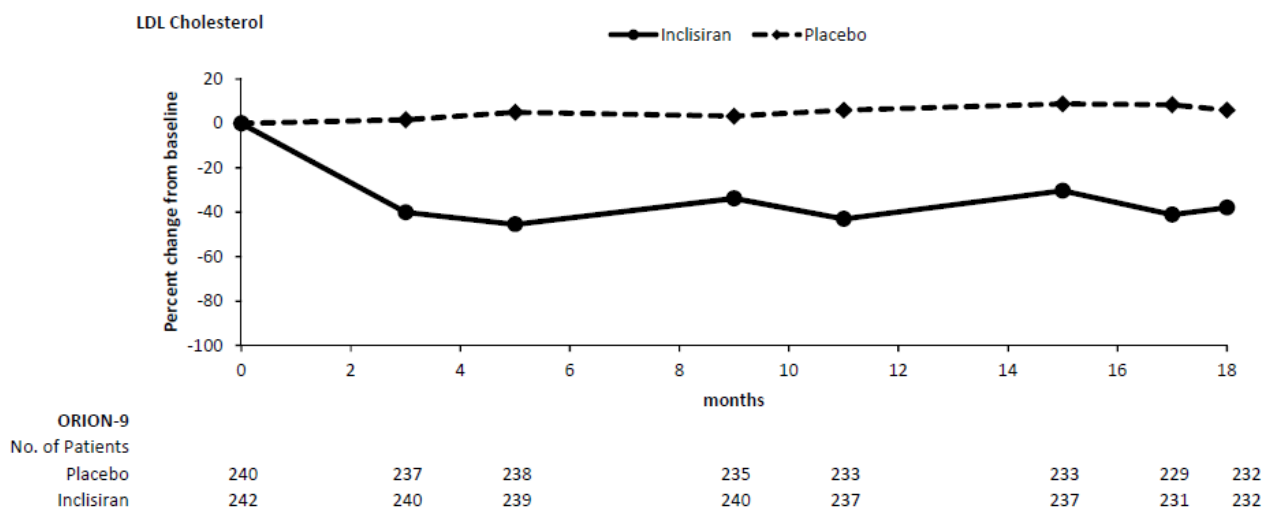
The primary efficacy outcome measure in Study 3 was the percent change from baseline to Day 510 in LDL-C. The difference between the LEQVIO and placebo groups in mean percentage change in LDL-C from baseline to Day 510 was -48% (95% CI: -54%, -42%;  $p < 0.0001$ ). For additional results, see Table 4 and Figure 3.

**Table 4: Changes in Lipid Parameters in Patients with HeFH on Maximally Tolerated Statin Therapy (Mean % Change from Baseline to Day 510 in Study 3)**

Treatment Group	LDL-C	Total Cholesterol	Non-HDL-C	ApoB
Day 510 (mean percentage change from baseline)				
Placebo (n = 240)	8	7	7	3
LEQVIO (n = 242)	-40	-25	-35	-33
Difference from placebo (LS Mean) (95% CI)	-48 (-54, -42)	-32 (-36, -28)	-42 (-47, -37)	-36 (-40, -32)

ApoB = apolipoprotein B; CI = confidence interval; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol

4.5% of subjects on LEQVIO and 4.6% of subjects on placebo had missing LDL-C data at primary endpoint (Day 510). Missing data were imputed using a modified control-based multiple imputation to account for treatment adherence. Percent change from baseline in LDL-C was analyzed using analysis of covariance (ANCOVA) with fixed effect for treatment group and baseline LDL-C as a covariate. Other endpoints were analyzed using mixed-effect model for repeated measure (MMRM) with fixed effects for treatment group, visit, interaction between treatment and visit, and baseline value as a covariate. Missing data were imputed using a control-based pattern-mixture model approach.

**Figure 3: Mean Percent Change from Baseline in LDL-C Over 18 Months in Patients with HeFH on Maximally Tolerated Statin Therapy (Study 3)**

## 16 HOW SUPPLIED/STORAGE AND HANDLING

LEQVIO injection is a clear, colorless to pale yellow solution, 284 mg/1.5 mL (189 mg/mL) of inclisiran supplied as:

Carton containing 1 single-dose prefilled syringe.

NDC 0078-1000-60

Store LEQVIO at controlled room temperature 20°C to 25°C (68°F to 77°F) with allowable excursions between 15°C and 30°C (59°F and 86°F) [see USP, Controlled Room Temperature (CRT)].

## 17 PATIENT COUNSELING INFORMATION

### Pregnancy

Advise pregnant patients and patients who can become pregnant of the potential risk to a fetus. Advise patients to inform their healthcare provider of a known or suspected pregnancy to discuss if LEQVIO should be discontinued [*see Use in Specific Populations (8.1)*].

### Injection Site Reactions

Advise patients that injection site reactions can occur with LEQVIO.

Distributed by:

Novartis Pharmaceuticals Corporation  
East Hanover, New Jersey 07936

For more information, visit [www.leqvio.com](http://www.leqvio.com) or call 1-833-LEQVIO2 (1-833-537-8462).

© Novartis

T2021-148

# Exhibit 42



## Parallel Structure

Parallel structure means that coordinate parts of a sentence, such as items in a series or list, have the same grammatical form. Items in a series must be all nouns, all verbs, or all participles, and so on. There are two reasons it is important to maintain parallelism in a series:

- 1) Sentences that maintain parallelism are much easier to read and process than those that do not. With parallel structure:

I like running, singing, and reading.

Without parallel structure:

I like running, singing, and to read. In the parallel structure, all of the objects of "like" are gerunds, -ing verbs acting as nouns. In the non-parallel structure, there are two gerunds acting as nouns and one infinitive (to read), which makes the sentence awkward and harder to process.

- 2) Maintaining parallelism helps writers avoid grammatically incorrect sentences. Below is an example of a grammatically incorrect sentence without parallel structure:

The computer's ability to multi-task, defend against viruses, and overall usability all improved when I updated the operating software.

The grammar error becomes clear when you make a bullet-point list of each item following the part of the sentence that each item is meant to complete:

The computer's ability to:

- Multi-task
- Defend
- Usability

"The computer's ability to usability improved" is a grammatically incorrect sentence because all words following the phrase "the computer's ability" must be verbs, but "usability" is a noun. By maintaining a parallel structure, you will avoid grammatically incorrect sentences containing lists.

If you are unsure if a sentence is parallel and has grammatically correct structure, try making a bullet-point list like the one above.

Writers should use parallel structure whether the items in the list or series are grammatical subjects, predicates, or objects.

Mowing the lawn, trimming the shrubs, and edging the sidewalk took her two hours to complete.

The writer interviewed two subject matter experts, edited four drafts, and composed two press releases.

On Saturdays they may visit the open-air market, the docks, or the park.

# Exhibit 43

**IN THE UNITED STATES DISTRICT COURT  
DISTRICT OF DELAWARE**

NIPPON SHINYAKU CO., LTD., Plaintiff,	)	
	)	
v.	)	
	)	
SAREPTA THERAPEUTICS, INC.,	)	C.A. No. 21-1015 (GBW)
Defendant.	)	
	)	
	)	
	)	
	)	
SAREPTA THERAPEUTICS, INC.,	)	
Defendant and Counter-Plaintiff	)	
	)	
v.	)	
	)	
NIPPON SHINYAKU CO., LTD. and	)	
NS PHARMA, INC., Plaintiff and Counter-	)	
Defendants.	)	
	)	

**DECLARATION OF MICHELLE L. HASTINGS, PH.D**

I, Michelle L. Hastings, declare as follows:

1. I have been asked by outside counsel for Nippon Shinyaku Co., Ltd. and NS Pharma, Inc. (collectively “NS”) to offer my opinions relating to certain claim construction disputes between the parties in connection with the above-referenced matter. I have been informed that this case has involved the following Asserted Patents, and that the parties dispute the meaning of certain terms used in those Asserted Patents:

No.	U.S. Patent No.
1	9,708,361 (“’361 Patent”)
2	10,385,092 (“’092 Patent”)
3	10,407,461 (“’461 Patent”)
4	10,487,106 (“’106 Patent”)
5	10,647,741 (“’741 Patent”)
6	10,662,217 (“’217 Patent”)
7	10,683,322 (“’322 Patent”)
8	9,994,851 (“’851 Patent”)
9	10,227,590 (“’590 Patent”)

10	10,266,827 (“827 Patent”)
----	---------------------------

I have been informed and understand that the first seven patents listed (the “NS Patents”) have been asserted by Nippon Shinyaku against Sarepta, and that the latter three patents listed (the “UWA Patents”) have been asserted by Sarepta against NS.

2. I submit this Declaration to provide relevant background information regarding the technology at issue in the Asserted Patents, and to set forth my opinion about the meaning of the disputed terms of the UWA Patents. If called as a witness to testify at a claim construction hearing, I expect to testify on the following topics and provide opinions and testimony on what is summarized in this declaration.

**I. QUALIFICATIONS, EXPERIENCE, AND PUBLICATIONS**

3. The following is a brief summary of my background and qualifications. My background and qualifications are more fully set out in my curriculum vitae (“CV”), attached as Exhibit 1 to this Declaration.

4. In 1992, I earned a Bachelors of Arts in Biology at St. Olaf College (Northfield, Minnesota). By 1998, I had completed my Ph.D in Biology at Marquette University (Milwaukee, Wisconsin). I underwent my postdoctoral training at Cold Spring Harbor Laboratory, a leading biological research institution, from 1998-2006 (Postdoctoral Fellow) and 2006-2007 (Senior Fellow).

5. In 2007, I became an Assistant Professor of Cell Biology and Anatomy, tenure-track at Chicago Medical School (CMS), School of Graduate and Postdoctoral Studies / Rosalind Franklin University of Medicine and Science (RFUMS). Since then, I was promoted to Associate Professor (2013), awarded tenure (2014), and promoted to Professor with tenure (2020) at RFUMS. Since 2018, I have been the Director of RFUMS’s Center for Genetic Disease, and since 2019 have been the Vice-Chair of RFUMS’s Cell Biology and Anatomy department. I have also

served as a Graduate Faculty Scholar at Northern Illinois University since 2017. In May 2023, I will begin a new position as a Professor of Pharmacology in the University of Michigan Medical School and Director of the new M-RNA Therapeutics program within the University of Michigan Biosciences Initiative, Center for RNA Biomedicine.

6. Throughout my career, my research has focused on understanding the genetic basis of disease and discovering new nucleic acid therapeutics that can modulate the process of pre-mRNA splicing to alter gene expression. My work has sought to develop effective means of targeting splicing with antisense oligonucleotides for the treatment of diseases such as Usher syndrome, cystic fibrosis, Batten, Alzheimer's and Parkinson's disease.

7. As my CV shows, I have roughly 60 peer-reviewed publications in this field since 1997, with many additional extramural invited presentations, oral presentations, and poster presentations. I am also named as inventor on 11 issued U.S. patents for antisense compounds.

8. I have received awards and recognition for my work, both internally within my research institution and externally from other organizations, including numerous awarded grants. Of particular note, this past year the National Academy of Sciences named me a Kavli Frontiers of Science Fellow.

9. I have been active in professional organizations in this field since graduate school, joining the RNA Society in 1996, as well as the American Society for Human Genetics in 2006, the American Society of Gene & Cell Therapy in 2018, and the Oligonucleotides Therapeutics Society in 2019. I have also served on multiple editorial boards for journals in this field, including Nucleic Acids Research (Editorial Board, 2015-present), the RNA Journal (2021-present), and RNA Therapeutics Society (Charter Board Member, 2022-present).

10. Throughout my career, I have also taken an interest in teaching and mentoring the next generation of scientists. Beyond regularly teaching coursework, I have supervised and mentored numerous post-doctoral fellows and research staff, as well as Ph.D, M.D., pharmacy, undergraduate, high school, and middle school students.

## **II. COMPENSATION**

11. I am being compensated for my time spent on this matter at my usual and customary rate of \$600 per hour, plus reasonable expenses. My compensation is not related to the outcome of this action, and I have no financial interest in the outcome of this case.

## **III. MATERIALS CONSIDERED**

12. In preparing this declaration, I have considered the materials identified in this declaration, as well as the following materials:

- the UWA Patents;
- the original prosecution histories of the UWA Patents;
- the parties' Joint Claim Construction Chart (December 15, 2022, D.I. 144) and accompanying appendix filed in this case;
- Sarepta's Opening Claim Construction Brief regarding the UWA Patents (as served January 5, 2023); and
- The Declaration of Dr. Cy A. Stein (as served January 5, 2023); and
- Any additional materials identified below.

13. I have also relied upon my decades of general experience in the field, though the testimony I offer is from the perspective of a person of ordinary skill as I have defined it below.

## **IV. THE LAW OF CLAIM CONSTRUCTION**

14. This section describes my understanding of currently applicable legal principles based on my conversations with counsel, which I have used in forming the opinions set forth

herein. I have used my understanding of these legal principles, as set forth in this section, to analyze the Asserted Patents.

15. I have been informed that claim construction is an issue of law for the Court to decide, although a particular claim construction may involve underlying factual issues that the Court must evaluate in reaching its determination. I am also informed that the construction of claims is a way of interpreting the claim language in order to understand and explain, but not to change, the scope of the claims.

16. I am informed that claim construction begins with a focus on the words of the claim themselves, and that the words of the claims are generally given their ordinary and customary meaning. The ordinary and customary meaning is the meaning that the term would have to a person of ordinary skill in the art in question at the time of the invention. I have been informed that a person of ordinary skill in the art is presumed to have read the patent's specification and prosecution history to better understand the context of the invention. I am also informed that in determining the ordinary meaning of a claim term, one should consult the intrinsic evidence, *e.g.*, the claims themselves, the specification, and the prosecution history, and to a lesser extent, extrinsic evidence, such as dictionaries, treatises, expert testimony.

17. I am also informed that where a patent derives from a parent application, such that they share the same written descriptions and use many common terms, the prosecution history of that parent application may be relevant to construing the patent at issue.

18. I am informed that the patent statute requires that a patent must "conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention." 35 U.S.C. § 112(b). I am also informed that some claims, despite being issued by the Patent Office, can later be found to be invalid if they

contain claim terms that are indefinite. I am informed that a “patent is invalid for indefiniteness if its claims, when read in light of the specification delineating the patent and the prosecution history, fail to inform, with reasonable certainty, those skilled in the art about the scope of the invention.” *Nautilus, Inc. v. Biosig Instruments, Inc.*, 134 S. Ct. 2124, 2129 (2014). Thus, I understand that indefiniteness is to be evaluated from the perspective of someone of ordinary skill in the relevant art at the time the patent was filed, reading the claims in light of the patent’s specification and prosecution history.

**V. PERSON OF ORDINARY SKILL IN THE ART (“POSA”)**

19. I understand from counsel that assessing the level of ordinary skill in an art may involve considering various factors, including the type of problems encountered in the art, prior art solutions to those problems, the rapidity with which innovations are made, the sophistication of the technology, and the educational level of active workers in the field.

20. Based on my review of the UWA Patents and experience in the field, I disagree somewhat with the definition offered by Dr. Stein. *See* Ex. 37 ¶ 26. Although I agree that a Ph.D. with relevant experience would qualify as a POSA, I do not see why an M.D., M.S., or B.S./B.A. would not also qualify if they had relevant experience. This is particularly true for the ’827 Patent, which has claims requiring administering an antisense oligonucleotide to a patient.

21. Thus, it is my opinion that a hypothetical POSA at the time of the inventions would be an individual that has an M.D., Ph.D. or lower degree with expertise in molecular biology, biochemistry or a related area, and experience with neuromuscular or genetic diseases and/or designing and testing antisense oligonucleotides for splice-site switching/exon skipping applications. The POSA would have general knowledge of antisense oligonucleotide chemical modifications to the backbone, nucleobases and other manipulations that can alter the activity of the antisense molecule, as well as delivery methods for antisense oligonucleotides. A POSA would

also have general knowledge regarding using antisense oligonucleotides in cell-free, cell-based and/or *in vivo* experiments, as well as DMD models and the use of antisense oligonucleotides to induce skipping of DMD exons to correct the open reading frame of the RNA transcripts. I have considered the issues below under both definitions, and reach the same opinions under both.

## VI. DISPUTED TERMS

22. I understand the parties have disputes regarding the meaning of the following terms used in asserted claims of the UWA Patents:

Term	Claim Term/Issue
1	“antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA”
1a	“a base sequence”
1b	Indefiniteness regarding “a target region”
1c	Indefiniteness regarding “exon 53 of the human dystrophin pre-mRNA”
2	Indefiniteness regarding “wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)”
3	Indefiniteness regarding “in which uracil bases are thymine bases”

23. For this declaration, I have been asked to provide (and herein provide) expert analysis of these terms of the UWA Patents, but reserve my right to provide analysis on the parties’ remaining disputes as needed, for example in response to any argument, or expert opinion(s), offered by Sarepta.

24. At the outset, it is worth noting that the claims of the UWA Patents are highly similar to each other, as illustrated below with claim 1 from each patent:

’851 Patent	’590 Patent	’827 Patent
1.	1.	1. <a href="#">A method for treating a patient with Duchenne muscular dystrophy (DMD) in need thereof who has a mutation of the DMD gene that is</a>

<p>An antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA, <u>wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)</u>, wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, and wherein the antisense oligonucleotide induces exon 53 skipping; or a pharmaceutically acceptable salt thereof.</p>	<p>An antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA,</p> <p>wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, and wherein the antisense oligonucleotide induces exon 53 skipping; or a pharmaceutically acceptable salt thereof.</p>	<p><u>amenable to exon 53 skipping, comprising administering to the patient</u> an antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA,</p> <p>wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, and wherein the antisense oligonucleotide induces exon 53 skipping; or a pharmaceutically acceptable salt thereof.</p>
--	---	--

Each claim recites “an antisense nucleotide of 20 to 31 bases comprising” certain characteristics. Compared to the other patents, the ’851 Patent’s claims require an additional “wherein” clause relating to the “target region” (Term 2, in Red), and the ’827 Patent’s claims contain a different preamble and require an “administering” step (in Blue).

25. Claim 2 of each UWA Patent implicates this structure as well. In each of the ’851 and ’590 Patents, claim 2 recites “A pharmaceutical composition comprising: (i) an antisense oligonucleotide” defined using the same language as in the respective claim 1, “and (ii) a pharmaceutical acceptable carrier.” In the ’827 Patent, claim 2 is a dependent claim that further recites “wherein the antisense oligonucleotide is administered intravenously.”

26. Given the commonalities among the asserted UWA Patents’ claims, my analysis below is often applicable across all claims. However, I address these differences below where relevant to my analysis. I also understand the specifications of the UWA Patents to be substantially the same, and therefore cite primarily to the ’851 Patent’s specification for convenience.

27. I have been informed by counsel and understand that NS initially identified Terms 1a, 1b, and 1c for construction, and that Sarepta proposed construing the broader Term 1 in response. After reviewing the parties' positions, I understand there to be three separate disputes within Term 1. Because considering Terms 1a, 1b, and 1c (and considering each subsidiary issue) informs my analysis of the broader Term 1, I discuss Terms 1a, 1b, and 1c first below.

28. As mentioned previously, I am informed that a patent is indefinite if the claims, read in light of the specification and the prosecution history, fail to inform, with reasonable certainty, a POSA about the scope of the invention. For the reasons discussed below, it is my opinion that the claims of the UWA Patents are indefinite because even when read in light of the specification and the prosecution history, they fail to inform a POSA about the scope of the claimed inventions with reasonable certainty.

## VII. Term 1a – “a base sequence”

29. The parties' proposed constructions for disputed Term 1a are shown below:

Claim Language	NS's Proposed Construction	Sarepta's Proposed Construction
“a base sequence”	“any sequence of bases that is part of the antisense oligonucleotide”	“a linear sequence of bases”

30. As highlighted below, using claim 1 of the '851 Patent as exemplary, Term 1a is found immediately after the language “antisense oligonucleotide of 20 to 31 bases comprising”:

1. An antisense oligonucleotide of 20 to 31 bases comprising **a base sequence** that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA, wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69), wherein **the base sequence** comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, and wherein the antisense oligonucleotide induces exon 53 skipping; or a pharmaceutically acceptable salt thereof.

The claim also refers back to “the base sequence” in a later-recited “wherein clause.”

31. In reviewing Dr. Stein’s declaration and Sarepta’s Opening Brief, the dispute here is whether the recited “base sequence” can only be the “entire linear sequence of bases” across “the overall length of an antisense oligonucleotide,” *see* Ex. 37 ¶ 51, or whether “any sequence of bases that is part of the antisense oligonucleotide” may qualify as a “base sequence.”

32. In my opinion, a POSA would recognize, based on the surrounding claim language, that the term “base sequence” encompasses any sequence of bases within the antisense oligonucleotide.

33. *First*, a POSA would find it significant that the word “comprising” follows the term “antisense oligonucleotide of 20 to 31 bases” and precedes the term “base sequence.” I have been informed and understand that in patent claims, “comprising” and “comprises” are a specialized terms meaning “including but not limited to.”<sup>1</sup> Thus, the claims effectively recite an “antisense oligonucleotide of 20 to 31 bases **including but not limited to** a base sequence that is . . . .” This would confirm to a POSA that the recited “base sequence” need only be a portion of the “antisense oligonucleotide,” and that the “antisense oligonucleotide” may or may not include additional bases or base sequences beyond the required “base sequence” it includes.

34. *Second*, a POSA would find it significant that the claim requires the “base sequence” to be “100% complementary to **consecutive bases of** a target region.” As discussed below, I do not believe the scope of “a target region” would be reasonably certain to a POSA. But regardless of what scope “target region” has, the claim here only requires complementarity to

---

<sup>1</sup> The specification also adopts this meaning. *See* Ex. 1, ’851 Patent at 23:10-14 (“Throughout this specification, unless the context requires otherwise, the word ‘comprise,’ or variations such as ‘comprises’ or ‘comprising,’ will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.”).

“consecutive bases of a target region,” rather than the “target region” as a whole. Put differently, the “base sequence” must be “100% complementary” to some portion of the “target region” that is consecutive. This likewise would indicate to a POSA that the term “base sequence” encompasses sequences of bases that make up less than the entire “antisense oligonucleotide.”

35. *Third*, the meaning that Dr. Stein and Sarepta attribute to Term 1a renders the claim language “comprising a base sequence” superfluous. If it is true, as Dr. Stein asserts, that only “the entire linear sequence of bases in an antisense oligonucleotide forms a base sequence, *i.e.*, the overall length of an antisense oligonucleotide is the same as the length of the base sequence itself,” Ex. 37 ¶ 51, then the claim means exactly the same as if the language “comprising a base sequence” were deleted:

An antisense oligonucleotide of 20 to 31 bases ~~comprising a base sequence~~ that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA . . . .

In fact, as discussed below, Sarepta’s collective construction for Term 1 omits the word “comprising.”

36. That the claims do use “base sequence” and “antisense oligonucleotide” as separate terms indicates to a POSA that “base sequence” carries a different meaning than the entire “antisense oligonucleotide.” The claims do not recite that the “**antisense oligonucleotide** of 20 to 31 bases . . . is 100% complementary”—they require (1) that the “antisense oligonucleotide of 20 to 31 bases compris[es] a base sequence”; and (2) that the “**base sequence** is 100% complementary.” This difference confirms to a POSA that the “base sequence” may be a subsidiary portion of the “antisense oligonucleotide,” and does not exclusively mean the entire length of the “antisense oligonucleotide.”

37. In my opinion, the specification further demonstrates that the claims use the term “base sequence” to mean any sequence of bases within the antisense oligonucleotide, and does not exclusively mean the entire “antisense oligonucleotide.”

38. According to the shared specification, “the sequence of an antisense molecule need **not** be 100% complementary to that of its target sequence to be specifically hybridisable.” ’851 Patent. at 25:26-28.<sup>2</sup> As it explains:

“[S]pecifically hybridisable” and “complementary” are terms which are used to indicate **a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs** between the oligonucleotide and the DNA or RNA target. . . . An antisense molecule is specifically hybridisable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is **a sufficient degree of complementarity to avoid non-specific binding** of the antisense compound to non-target sequences under conditions in which specific binding is desired . . . .

*Id.* at 25:22-35.

39. This disclosure supports my understanding of the claim language. The claims recite that the “antisense oligonucleotide” “comprises a base sequence”—a sequence of bases that is “100% complementary to consecutive bases of a target region” and “comprises at least 12 consecutive bases of . . . SEQ ID NO: 195”—to define a core set of bases within the “antisense oligonucleotide” that would ostensibly provide a “sufficient degree of complementarity or precise pairing” to make the antisense oligonucleotide “specifically hybridisable.” By contrast, the interpretation advanced by Sarepta and Dr. Stein would exclude these disclosed embodiments where a constituent sequence of 100% complementary bases within the antisense oligonucleotide makes the antisense oligonucleotide as a whole “specifically hybridizable.” As such, a POSA would understand this specification disclosure to support NS’s proposed construction.

---

<sup>2</sup> Emphasis added unless otherwise noted.

40. The specification’s disclosure of what it identifies as a “cunningly designed antisense oligonucleotide” termed a “weasel” also supports NS’s proposed construction. *See, e.g.*, ’851 Patent at 4:56-62 (discussing same, including an example of such antisense oligonucleotides in the prior art). As the shared specification explains, this type of “antisense oligonucleotide” is formed by “joining together two or more antisense oligonucleotide molecules.” *Id.* The resultant “antisense oligonucleotide” embodiments include sequences of bases that are each complementary to a different region of pre-mRNA. *Id.* Table 1C provides examples, including the following exon 53-related embodiment:

195	H53A(+23+47) -	CUG AAG GUG UUC UUG UAC UUC AUC C-
	AA-	
196	H53A(+150+175) -	UGU AUA GGG ACC CUC CUU CCA UGA CUC-
	AA-	AA-
<u>194</u>	H53D(+14-07)	UAC UAA CCU UGG UUU CUG UGA

A POSA would recognize that this embodiment is an “antisense oligonucleotide” that includes a base sequence that (1) comprises at least twelve consecutive bases of SEQ ID NO. 195 (namely, all twenty-five bases) and (2) is 100% complementary to consecutive bases of a portion of pre-mRNA (namely, the bases at h53A(+23+47) of the wild-type dystrophin pre-mRNA transcript).

41. Confusingly, Sarepta’s and Dr. Stein’s interpretation of the “base sequence” limitations would exclude this embodiment simply because it contained additional base sequences beyond SEQ ID NO. 195.<sup>3</sup> This is inconsistent with the specification’s articulated meaning of “comprising”—that it “impl[ies] the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.” ’851 Patent at 23:10-14.

---

<sup>3</sup> According to the specification, this embodiment is a “2’-O-methyl phosphorothioate antisense oligonucleotide[],” not a morpholino, and its length exceeds 31 bases. *See, e.g.*, 851 Patent at Table 1C. While it would not meet those limitations of the asserted claims, it is nevertheless instructive about the analogous shorter, morpholino oligonucleotides that Sarepta’s and Dr. Stein’s interpretation would exclude.

42. Dr. Stein argues that NS's proposed construction "conflicts with the specification" because in Table 1A, the "base sequence of each exemplary antisense oligonucleotide includes *all* of the bases contained within the antisense oligonucleotide, not just some portion of them." Ex. 37 ¶¶ 50-51, 69. But there is no conflict. NS's proposed construction means that "base sequence" encompasses "any sequence of bases that is part of the antisense oligonucleotide." A sequence of bases that consists of "all of the bases contained within the antisense oligonucleotide" obviously meets that definition, such that these examples comport with NS's proposal.

43. In any event, Dr. Stein's logic does not consider the entire specification. According to Dr. Stein, the 21 bases that make up SEQ ID No. 194 (UAC UAA CCU UGG UUU CUG UGA), the 25 bases that make up SEQ ID No. 195 (CUG AAG GUG UUC UUG UAC UUC AUC C), and the 27 bases that make up SEQ ID No. 196 (UGU AUA GGG ACC CUC CUU CCA UGA CUC) would each qualify as a "base sequence" because they are each separately listed in Table 1A. *See, e.g.*, Ex. 37 ¶ 69. But, as discussed, each of these same base sequences is also listed in Table 1C as a constituent portion of a "weasel"-style "antisense oligonucleotide." Taking the specification as a whole thus demonstrates that the claim term "base sequence" encompasses both constituent base sequences **and** entire base sequences.

44. Additionally, in my opinion, if Sarepta's proposal for Term 1a ("a linear sequence of bases") is input into the claim after "comprising," it would not carry the meaning Dr. Stein and Sarepta attach to "base sequence." Rather, it would carry the same meaning as NS's proposed construction. A sequence of bases can be "linear" without spanning an entire antisense oligonucleotide, such that an "antisense oligonucleotide of 20 to 31 bases comprising a linear sequence of bases" would still mean that the "antisense oligonucleotide" includes the "linear sequence of bases" but does not exclude other bases or base sequences. *See, e.g.*, '851 Patent at

23:10-14. To carry the meaning Dr. Stein and Sarepta propose, Sarepta’s construction for Term 1a would need to be modified to clarify that the claim term is limited to the sequence of bases spanning the **entire** antisense oligonucleotide, and excludes all other sequences of bases. Indeed, Dr. Stein uses such additional language when stating his opinion. *See, e.g.*, Ex. 37 ¶ 51 (referring to “the entire linear sequences of bases in an antisense oligonucleotide”). As discussed above, however, such an exclusionary construction would be inconsistent with the claim language and the shared specification’s disclosures.

45. I have also reviewed the prosecution history of the UWA Patents cited by the parties (as listed in Exhibit B to the Joint Claim Construction Chart). In my opinion, these excerpts provide additional support for NS’s proposed construction, as they contain examples where the Office and Applicant refer to a “sequence” of bases spanning less than the entire antisense oligonucleotide.

46. When prosecuting the ’851 Patent, the Office initially rejected the claims as obvious over van Ommen (WO 2004/083432) and Koenig et al. (Nature 338, 509-511, Apr. 6, 1989). *See* Ex. 22, at SRPT-VYDS-0004608-11. In response, the Applicant acknowledged the Office’s reliance on “SEQ ID NO: 29 (h53AON1), which [the Office] contends is a 18-mer oligonucleotide having a sequence identical to three nucleotides of SEQ ID NO: 195.” *Id.* at SRPT-VYDS-0004785. This statement uses the same meaning for “sequence” that NS proposes for Term 1a—the Applicant describes the Office as contending that the prior art antisense oligonucleotide spanning 18 bases includes a constituent “sequence” spanning only 3 bases that is “identical” a corresponding portion of SEQ ID NO: 195.

47. As another example, the Applicant’s response also describes an argument by the Office that “the skilled artisan would ‘try’ to enhance activity by ‘a common and efficient strategy’

of synthesizing and testing ‘longer oligonucleotides **containing within them the sequence** known to have the desired activity.’” *Id.* at SRPT-VYDS-0004789. Again, this statement supports NS’s proposed construction, and demonstrates the Office’s belief that an “antisense oligonucleotide” may include both a defined “sequence” and additional bases beyond that “sequence.”

48. Dr. Stein appears to argue that a statement made by the Office in Interference No. 106,007 (which related to a separate Wilton patent than the ’851, ’590, and ’827 Patents at issue here) supports Sarepta’s view of the claim. *See* Ex. 37 ¶ 57 (quoting the statements “an oligonucleotide that includes a nucleobase sequence that is complementary to a portion of a particular pre-mRNA exon” and “exon 53 of the pre-mRNA associated with the gene responsible for the formation of the protein dystrophin”). I have reviewed this statement in its original context, *see* Ex. 17 at 4, and disagree with Dr. Stein. This statement reflects NS’s proposed construction in that the oligonucleotide “includes” the nucleobase sequence—it does not say that the oligonucleotide is limited to having only the nucleobase sequence.

49. Regardless, even if Dr. Stein were correct about this particular statement, it would not change my overall opinion regarding Term 1a. As discussed above, other specification disclosures and prosecution history show that a “base sequence” does not **have to** span the entire oligonucleotide (but certainly could).

50. For all the reasons discussed above, in my opinion a POSA would conclude from the intrinsic and extrinsic evidence that the term “base sequence” encompasses “any sequence of bases that is part of the antisense oligonucleotide.”

#### **VIII. Term 1b – “a target region”**

51. The parties’ positions for disputed Term 1b are shown below:

Claim Language	NS's Proposed Construction	Sarepta's Proposed Construction
"a target region"	<i>Indefinite</i>	"a segment of the pre-mRNA"

52. As highlighted below, using claim 1 of the '851 Patent as exemplary, Term 1b is found immediately after the language "consecutive bases of":

1. An antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of **a target region** of exon 53 of the human dystrophin pre-mRNA, wherein **the target region** is within annealing site H53A(+23+47) and annealing site H53A(+39+69), wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, and wherein the antisense oligonucleotide induces exon 53 skipping; or a pharmaceutically acceptable salt thereof.

The '851 Patents' claims also refer back to "the target region" in a later-recited "wherein clause."

53. In my opinion, a POSA reviewing the UWA Patents' claims would be immediately uncertain regarding what "target region" means here. To be sure, the surrounding claim language indicates that "target region" would be some portion of some human, exon 53 pre-mRNA, but a POSA would not be reasonably certain regarding what portion(s) of the pre-mRNA must be "targeted" to satisfy this claim language.<sup>4</sup>

54. In my experience, POSAs at the time of the invention (and still to this day) did not consistently use "target" to refer to any specific sequence of pre-mRNA. The shared specification actually illustrates this, as it refers to "targeting" a portion of pre-mRNA in at least three separate

---

<sup>4</sup> In the '851 Patent, Term 2 requires the "target region" to be "within annealing site H53A(+23+47) and annealing site H53A(+39+69)." As discussed below in connection with Term 2, this narrows the location of the "target region" in the '851 Patent to being "within" a 9-base span of exon 53. But it does not answer the question of what pre-mRNA feature a "target region" actually is, *i.e.*, what pre-mRNA feature is being "targeted" within that 9-base location.

ways: (1) to generally identify the exon generally being targeted (*e.g.*, exon 53 v. another exon); (2) to refer to the particular motifs or regulatory regions on a pre-mRNA transcript being targeted (*e.g.*, acceptor site, donor site, enhancers, silencers); and (3) to refer to the exact bases of pre-mRNA to which an antisense oligonucleotide anneals (or binds).

55. Each of these three separate possible meanings for “target region” are exemplified in the specification’s discussion of the inventors’ chosen nomenclature:

This nomenclature became especially relevant when testing **several slightly different antisense molecules, all directed at the same target region**, as shown below:

$H\#A/D(x,y)$ .

The first letter designates the species (*e.g.* H: human, M: murine, C: canine) “#” designates **target dystrophin exon** number.

“A/D” indicates **acceptor or donor splice site** at the beginning and end of the exon, respectively.

(x y) represents the **annealing coordinates** where “—” or “+” indicate intronic or exonic sequences respectively. As an example, A(-6+18) would indicate the last 6 bases of the intron preceding the target exon and the first 18 bases of the target exon. The closest splice site would be the acceptor so these coordinates would be preceded with an “A”. Describing annealing coordinates at the donor splice site could be D(+2-18) where the last 2 exonic bases and the first 18 intronic bases correspond to the annealing site of the antisense molecule. Entirely exonic annealing coordinates that would be represented by A(+65+85), that is the site between the 65th and 85th nucleotide from the start of that exon.

’851 Patent at 22:44-65. As emphasized above, this disclosure first notes that “several slightly different antisense molecules” may “all [be] directed at the same target region.” Thus, in this sentence, the specification uses “target region” to describe some broader pre-mRNA region beyond the exact annealing site of a given antisense oligomer. The passage then identifies two different types of broader pre-mRNA regions: the “target dystrophin exon,” and regulatory motifs associated with given exons (“acceptor or donor splice site”). And it last describes the “annealing coordinates,” *i.e.*, the exact “annealing site of the antisense molecule.”

56. The rest of the specification likewise uses “target” with these three different meanings. As shown below, the specification repeatedly uses “target” to generally identify the exon being targeted:

With some **targets such as exon 19**, antisense oligonucleotides as short as 12 bases were able to induce exon skipping, albeit not as efficiently as longer (20-31 bases) oligonucleotides. In some **other targets, such as murine dystrophin exon 23**, antisense oligonucleotides only 17 residues long were able to induce more efficient skipping than another overlapping compound of 25 nucleotides.

*Id.* at 23:63-24:3.

In other **exons targeted for removal**, masking the donor splice site did not induce any exon skipping. However, by directing antisense molecules to the acceptor splice site (human exon 8 as discussed below), strong and sustained exon skipping was induced.

*Id.* at 24:21-25.

This is not an isolated instance as the same effect is observed in canine cells where **targeting exon 8 for removal** also resulted in the skipping of exon 9. **Targeting exon 23 for removal** in the mouse dystrophin pre-mRNA also results in the frequent removal of exon 22 as well.

*Id.* at 24:31-35.

57. As shown below, the specification repeatedly uses “target” to refer to targeting particular motifs or regulatory elements:

Dunkley et al., (1997) Nucleosides & Nucleotides, 16, 1665-1668 described in vitro constructs for analysis of splicing around exon 23 of mutated dystrophin in the mdx mouse mutant, a model for muscular dystrophy. Plans to analyse these constructs in vitro using 2' modified oligonucleotides **targeted to splice sites** within and adjacent to mouse dystrophin exon 23 were discussed, though no target sites or sequences were given.

'851 Patent at 3:22-29.

An antisense oligonucleotide **targeted to the 3' splice site** of murine dystrophin intron 22 was reported to cause skipping of the mutant exon as well as several flanking exons and created a novel in-frame dystrophin transcript with a novel internal deletion.

*Id.* at 3:32-35.

Wilton et al, (1999), also describe **targeting the acceptor region** of the mouse dystrophin pre-mRNA with longer antisense oligonucleotides and being unable to repeat the published results of Dunckley et al., (1998).

*Id.* at 3:67-4:3.

The choice of target selection plays a crucial role in the efficiency of exon skipping and hence its subsequent application of a potential therapy. Simply designing antisense molecules to **target regions** of pre-mRNA presumed to be involved in splicing is no guarantee of inducing efficient and specific exon skipping. The most obvious or readily defined targets for splicing intervention are **the donor and acceptor splice sites** although there are less defined or conserved motifs including **exonic splicing enhancers, silencing elements and branch points**.

*Id.* at 4:30-38.

When antisense molecule(s) are **targeted to nucleotide sequences involved in splicing** in exons within pre-mRNA sequences, normal splicing of the exon may be inhibited causing the splicing machinery to by-pass the entire mutated exon from the mature mRNA.

*Id.* at 23:24-28.

As reported in Mann et al., (2002) there was a significant variation in the efficiency of bypassing the nonsense mutation depending upon antisense oligonucleotide annealing (“Improved antisense oligonucleotide induced exon skipping in the mdx mouse model of muscular dystrophy”. *J Gen Med* 4: 644-654). **Targeting the acceptor site** of exon 23 or **several internal domains** was not found to induce any consistent exon 23 skipping.

*Id.* at 24:12-20.

Within the context of the present invention, **preferred target site(s)** are those involved in mRNA splicing (i.e. **splice donor sites, splice acceptor sites or exonic splicing enhancer elements**). **Splicing branch points and exon recognition sequences or splice enhancers are also potential target sites** for modulation of mRNA splicing.

*Id.* at 24:54-60. These regulatory regions or motifs are often important sequences for modulating splicing activity, as they represent locations where the proteins involved in splicing interact with the pre-mRNA transcript.

58. The specification also repeatedly uses “target” to refer to the particular annealing sites of antisense oligonucleotides:

According to a first aspect, the invention provides antisense molecules capable of binding to **a selected target** to induce exon skipping.

*Id.* at 4:44-46.

The present invention describes antisense molecules capable of **binding to specified dystrophin pre-mRNA targets** and re-directing processing of that gene.

#### Antisense Molecules

According to a first aspect of the invention, there is provided antisense molecules capable of **binding to a selected target** to induce exon skipping.

*Id.* at 23:38-45.

**Annealing sites** on the human dystrophin pre-mRNA were selected for examination, initially based upon known or predicted motifs or regions involved in splicing. **2OMe antisense oligonucleotides were designed to be complementary to the target sequences** under investigation and were synthesised on an Expedite 8909 Nucleic Acid Synthesiser.

*Id.* at 32:31-36.

59. Yet further disclosures describe “targeting” or “directing” antisense oligonucleotides in multiple of these meanings in quick succession:

Thus, there remains a need to provide antisense oligonucleotides capable of binding to and modifying the splicing of a **target nucleotide sequence**. Simply **directing the antisense oligonucleotides to motifs** presumed to be crucial for splicing is no guarantee of the efficacy of that compound in a therapeutic setting.

’851 Patent at 4:17-22 (using both the regulatory element and annealing site meanings).

In other **targeted exons**, antisense molecules **directed at the donor or acceptor splice sites** did not induce exon skipping while annealing antisense molecules to intra-exonic regions (i.e. exon splicing enhancers within human dystrophin exon 6) was most efficient at inducing exon skipping. Some exons, both mouse and human exon 19 for example, are readily skipped **by targeting antisense molecules to a variety of motifs**. That is, targeted exon skipping is induced after using antisense oligonucleotides to mask donor and acceptor splice sites or exon splicing enhancers.

*Id.* at 24:38-47 (using both the general exon and regulatory element meanings).

This data shows that some particular antisense molecules induce efficient exon skipping while **another antisense molecule, which targets a near-by or**

**overlapping region**, can be much less efficient. Titration studies show one compound is able to induce targeted exon skipping at 20 nM while the less efficient antisense molecules only induced exon skipping at concentrations of 300 nM and above. Therefore, we have shown that **targeting of the antisense molecules to motifs involved in the splicing process** plays a crucial role in the overall efficacy of that compound.

Efficacy refers to the ability to induce consistent skipping of **a target exon**. However, sometimes skipping of **the target exons** is consistently associated with a flanking exon. That is, we have found that the splicing of some exons is tightly linked. For example, in **targeting exon 23** in the mouse model of muscular dystrophy with antisense molecules **directed at the donor site** of that exon, dystrophin transcripts missing exons 22 and 23 are frequently detected. As another example, when using an antisense molecule **directed to exon 8** of the human dystrophin gene, all induced transcripts are missing both exons 8 and 9. Dystrophin transcripts missing only exon 8 are not observed.

*Id.* at 33:53-34:20 (using all three meanings).

60. The different meanings result in different claim scope. if “target region” refers to the regulatory elements involved with splicing, then Term 1b imposes a requirement that the “consecutive bases” on the pre-mRNA to which the “base sequence” is “100% complementary” be from such an element. As the specification makes clear, this may or may not be the case. *See, e.g.,* ’851 Patent at 24:18-20 (noting that targeting “several internal domains” of exon 23 “was not found to induce any consistent exon 23 skipping). As is well-known in the art (and illustrated in Figure 1), not every base of a given pre-mRNA transcript is associated with a regulatory motif:

### FIGURE 1

bp                      Acceptor                      ESE                      Donor

ucaugcacugagugacccuuucucgcagGCGCUAGCUGGAGCA/////CCGUGCAGACUGACGgucucau

SEQ ID NO:213

SEQ ID NO:214

61. By contrast, if, as Dr. Stein opines, “target region” refers to the segment of pre-mRNA of exon 53 to which a given antisense oligomer happens to anneal, *see* Ex. 37 ¶ 37 (“An

antisense oligonucleotide is a short chain of nucleotides designed to target a specific portion of the pre-mRNA (often referred to as a ‘target region’) by having a base sequence that is complementary to the target region.”), ¶ 48 (quoting three instances where the specification uses the annealing site meaning),<sup>5</sup> then Term 1b would impose a requirement tailored to the specific antisense oligonucleotide binding site, and the claims would have different scope.

62. In my opinion, Dr. Stein’s opinion that “target region” definitively has the annealing site meaning is flawed. Dr. Stein’s analysis does not identify—much less attempt to reconcile—the different contexts in which the shared specification describes “targeting” pre-mRNA that I have discussed above. In fact, his own declaration follows the same inconsistent use of “target” as the specification. *Compare id.* ¶ 36 (discussing skipping of “the targeted exon”), *with id.* ¶ 37 (adopting the annealing site meaning for “target region”).

63. But perhaps more importantly, Dr. Stein does not recognize that the specification **only** uses the exact language “target region” twice, and that neither of those uses apply the annealing site meaning he adopts. In the first, the specification states that its “nomenclature became especially relevant when testing several slightly different antisense molecules, all directed at the same **target region** . . . .” ’851 Patent at 22:44-46. As discussed above, this use of “target region” describes some broader pre-mRNA region to which multiple “different antisense molecule” are annealing. In the second passage, the specification uses “target region” with an identification of regulatory elements in splicing (“donor and acceptor splice sites,” “exonic

---

<sup>5</sup> I disagree that the passage at column 24, lines 48 to 60 uses the annealing site meaning. This passage states that the “preferred target site(s) are those involved in splicing (i.e., **splice donor sites, splice acceptor sites, or exonic splicing enhancer elements**). **Splicing branch points and exon recognition sequences or splice enhancers** are also potential target sites for modulation of mRNA splicing.” Each of these listed “target sites” are regulatory elements in splicing.

splicing enhancers, silencing elements and branch points”) as “[t]he most obvious or readily defined targets”:

The choice of target selection plays a crucial role in the efficiency of exon skipping and hence its subsequent application of a potential therapy. Simply designing antisense molecules to **target regions** of pre-mRNA presumed to be involved in splicing is no guarantee of inducing efficient and specific exon skipping. The most obvious or readily defined targets for splicing intervention are **the donor and acceptor splice sites** although there are less defined or conserved motifs including **exonic splicing enhancers, silencing elements and branch points**.

*Id.* at 4:30-38. Thus, from the available options, in my opinion, a POSA would not find it reasonably certain that the claim language “target region” has the annealing site meaning.

64. I have also reviewed the prosecution history of the UWA Patents cited by the parties (as listed in Exhibit B to the Joint Claim Construction Chart). In my opinion, the prosecution history does not resolve the uncertainty a POSA would have regarding Term 1b and the scope of the claims.

65. In short, the prosecution history continues to use “target” with reference to pre-mRNA in different ways. As an example, the Office’s initial obviousness rejection for the ’851 Patent states that “van Ommen et al. has identified exon 53 and shown oligonucleotides **targeting this region** can cause exon skipping,” which uses “target” to refer to a particular exon. Ex. 22 at SRPT-VYDS-0004610. The Applicant’s later response, however, cites literature stating that “there is great variability for different **targets** and exons,” which uses “target” to refer to something other than a particular exon. *Id.* at SRPT-VYDS-0004793. On the following page, however, Applicant refers to antisense oligonucleotides that “**target** exon 50,” again using “target” to refer to a particular exon. *Id.* at SRPT-VYDS-0004794.

66. If anything, this inconsistent usage of “target” in the prosecution history would exacerbate a POSA’s uncertainty regarding the meaning of “target region” and scope of the claims.

67. To summarize, the intrinsic evidence provides competing meanings for this term, without providing reasonable certainty regarding which meaning the claims apply. Thus, in my opinion, the claims of the UWA Patents do not inform those skilled in the art about the scope of the claimed inventions with reasonable certainty due to their recitation of “a target region.”

**IX. Term 1c – “exon 53 of the human dystrophin pre-mRNA”**

68. The parties’ positions for disputed Term 1c are shown below:

Claim Language	NS’s Proposed Construction	Sarepta’s Proposed Construction
“exon 53 of the human dystrophin pre-mRNA”	<i>Indefinite</i>	“the pre-mRNA transcribed from exon 53 of the human dystrophin gene”

69. As highlighted below, using claim 1 of the ’827 Patent as exemplary, Term 1c is found immediately after the language “a target region of”:

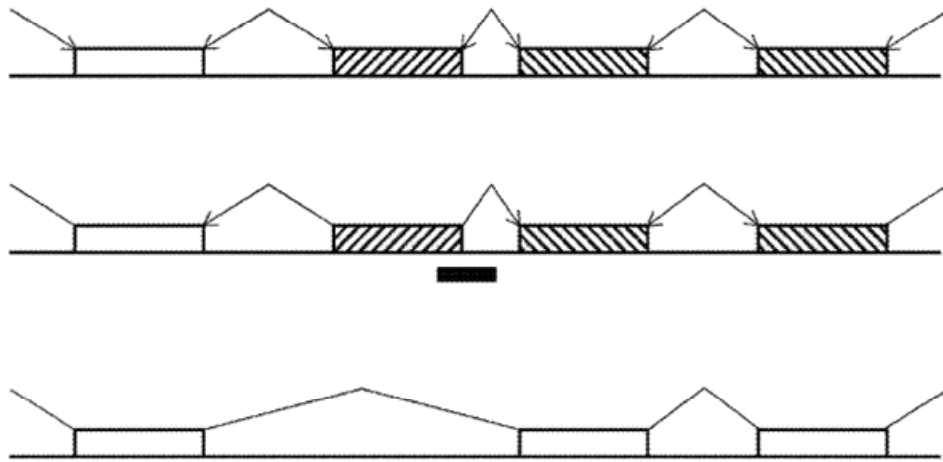
1. A method for treating a patient with Duchenne muscular dystrophy (DMD) in need thereof who has a mutation of the DMD gene that is amenable to exon 53 skipping, comprising administering to the patient an antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of **exon 53 of the human dystrophin pre-mRNA**, wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, and wherein the antisense oligonucleotide induces exon 53 skipping; or a pharmaceutically acceptable salt thereof.

70. As Dr. Stein notes, the dystrophin gene was identified in the 1980s, and subsequent research has identified the typical nucleotide sequences associated with its various exons, including exon 53. *See, e.g.*, Ex. 37 ¶ 34; *see also* Ex. 47; Ex. 18. Normal (or “consensus”) sequences for an entire gene are commonly called the “wildtype” gene.

71. DMD patients do not have a wildtype dystrophin gene. As Dr. Stein recognizes, DMD patients instead have “mutations in the[ir] dystrophin gene.” Ex. 37 ¶ 35; *see also* ’851

Patent at 24:64-25:2 (“Duchenne muscular dystrophy arises from mutations that preclude the synthesis of a functional dystrophin gene product. These Duchenne muscular dystrophy gene defects are typically nonsense mutations or genomic rearrangements such as deletions, duplications or microdeletions or insertions that disrupt the reading frame.”). “[T]here are many positions where these mutations can occur.” *Id.* at 25:2-6.

72. As the shared specification explains, the general logic behind exon skipping technology is that “if **exons associated with disease-causing mutations** can be specifically deleted from some genes, a shortened protein product can sometimes be produced that has similar biological so properties of the native protein or has sufficient biological activity to ameliorate the disease caused by mutations associated with the target exon.” ’851 Patent at 2:42-54. This concept is illustrated by Figure 2, which shows the antisense oligonucleotide annealing to a mutated exon to cause it to be “skipped” during splicing:



**FIGURE 2**

*See also id.* at 5:35-40 (“The hatched box represents an exon carrying a mutation that prevents the translation of the rest of the mRNA into a protein. The solid black bar represents an antisense oligonucleotide that prevents inclusion of that exon in the mature mRNA.”).

73. With this background in mind, in my opinion, a POSA would immediately find it unclear whether the claim term “exon 53 of the human dystrophin pre-mRNA” refers to exon 53 from wildtype pre-mRNA or patient’s mutated pre-mRNA.

74. Unfortunately, the context provided by surrounding claim language does not resolve this uncertainty. In the ’851 and ’590 Patents, for example, the claims do not provide any antecedent basis for “the human dystrophin pre-mRNA”—Term 1c is the first (and only) time those claims recite pre-mRNA.<sup>6</sup> As such, the claim language does not indicate one way or the other whether the claims are reciting a wildtype or mutated pre-mRNA.

75. The ’827 Patent only exacerbates this uncertainty. Despite its claims’ similarity to the ’851 and ’590 Patents, they additionally recite “a patient with Duchenne muscular dystrophy (DMD) in need thereof who has a mutation of the DMD gene that is amenable to exon 53 skipping.” Thus, while the ’827 Patent’s claims still do not expressly recite “pre-mRNA” outside of Term 1c, they do describe a mutated dystrophin gene “amenable to exon 53 skipping,” and thus suggest a mutated pre-mRNA.

76. The shared specification does not resolve this uncertainty either, as it describes experiments conducted with both wildtype and mutated cell lines. For example, the specification’s Background Section describes a 1995 study by Takeshima et al. that induced skipping of in dystrophin minigenes corresponding to “wild-type pre-mRNA”:

---

<sup>6</sup> I have been informed by counsel and understand that in patent claiming, claim terms are typically introduced using an indefinite article (e.g., “a” or “an”), and subsequent use of definite articles (e.g., “the” or “said”) typically refers back to a previously-recited claim term.

For example, modulation of mutant dystrophin premRNA splicing with antisense oligoribonucleotides has been reported both in vitro and in vivo. In one type of dystrophin mutation reported in Japan, a 52-base pair deletion mutation causes exon 19 to be removed with the flanking introns during the splicing process (Matsuo et al., (1991) J Clin Invest., 87:2127-2131). An in vitro minigene splicing system has been used to show that a 31-mer 2'-O-methyl oligoribonucleotide complementary to the 5' half of the deleted sequence in dystrophin Kobe exon 19 **inhibited splicing of wild-type pre-mRNA** (Takeshima et al. (1995), J. Clin. Invest., 95, 515-520).

'851 Patent at 3:8-21. But the immediately following disclosure describes experiments conducted using mutated dystrophin model:

Dunckley et al., (1997) Nucleosides & Nucleotides, 16, 1665-1668 described in vitro constructs for analysis of splicing around exon 23 of mutated dystrophin **in the mdx mouse mutant, a model for muscular dystrophy**. Plans to analyse these constructs in vitro using 2' modified oligonucleotides targeted to splice sites within and adjacent to mouse dystrophin exon 23 were discussed, though no target sites or sequences were given.

2'-O-methyl oligoribonucleotides were subsequently reported to correct dystrophin deficiency in myoblasts from the mdx mouse from this group. An antisense oligonucleotide targeted to the 3' splice site of murine dystrophin intron 22 **was reported to cause skipping of the mutant exon** as well as several flanking exons and created a novel in-frame dystrophin transcript with a novel internal deletion. This mutated dystrophin was expressed in 1-2% of antisense treated mdx myotubes. Use of other oligonucleotide modifications such as 2'-O-methoxyethyl phosphodiester are described (Dunckley et al. (1998) Human Mol Genetics, 5, 1083-90)

*Id.* at 3:22-40.

77. Later, the specification describes a series of experiments conducted by the inventors using normal human tissue, *i.e.*, the wildtype pre-mRNA:

Briefly, **normal primary myoblast cultures** were prepared from human muscle biopsies obtained after informed consent. The cells were propagated and allowed to differentiate into myotubes using standard culturing techniques. The cells were then transfected with the antisense oligonucleotides by delivery of the oligonucleotides to the cells as cationic lipoplexes, mixtures of antisense molecules or cationic liposome preparations.

*Id.* at 32:48-55. But, of course, the specification also states that the antisense oligonucleotides of the invention are for use in patients with mutated genes:

According to a third aspect, the invention provides a method for treating **a patient suffering from a genetic disease wherein there is a mutation in a gene encoding a particular protein** and the affect of the mutation can be abrogated by exon skipping, comprising the steps of: (a) selecting an antisense molecule in accordance with the methods described herein; and (b) administering the molecule to a patient in need of such treatment

*Id.* at 5:1-7.

78. I have also reviewed the prosecution history of the UWA Patents cited by the parties (as listed in Exhibit B to the Joint Claim Construction Chart). In my opinion, the prosecution history does not resolve the uncertainty a POSA would have regarding Term 1c and the scope of the claims. Like the specification, the prosecution history notes a series of studies, some of which use mutated cell lines, whereas others use normal cells. *See, e.g.*, Ex. 22 at SRPT-VYDA-0004791-92 (discussing Mann et al., J. Gene Med., 4(6):644-654 (2002)), SRPT-VYDS-0004794 (discussing Wu et al.’s use of “normal human myoblasts”); Ex. 19 at 647 (noting that both “normal and *mdx* cells were cultured”).

79. The intrinsic evidence therefore leaves a POSA with two possible interpretations for the meaning of “exon 53 of the human dystrophin pre-mRNA,” each with a different resulting claim scope. If Term 1c refers to the wildtype pre-mRNA, then the “complementary” limitation of the asserted claims will be evaluated against the wildtype pre-mRNA sequence. But if it refers to a patient’s mutated pre-mRNA, that limitation will be evaluated against the mutated pre-mRNA sequence for exon 53, which may or may not differ from the wildtype sequence, depending on the patient. *See, e.g.*, ’851 Patent at 2:42-54 (explaining that skipping can provide relief “if exons associated with disease-causing mutations can be specifically deleted”), 5:35-40 (illustrating an antisense oligonucleotide annealing to “an exon carrying a mutation”).

80. Thus, in my opinion, the claims of the UWA Patents do not inform those skilled in the art about the scope of the claimed inventions with reasonable certainty due to each of their recitation of “exon 53 of the human dystrophin pre-mRNA.”

**X. Term 1 – “antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA”**

81. The parties’ proposed constructions for disputed Term 1 are shown below:

Claim Language	NS’s Proposed Construction	Sarepta’s Proposed Construction
“antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA”	<i>Indefinite;</i>  Or, in the alternative:  “antisense oligonucleotide with 20 to 31 bases that includes any sequence of bases that is part of the antisense oligonucleotide that are 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA”	<i>Not indefinite.</i>  <i>No construction needed in light of inter alia, the intrinsic evidence and the knowledge of a person of ordinary skill in the art</i>  To the extent construction is needed, Sarepta proposes that the phrase should be given its plain and ordinary meaning, i.e.,  “antisense oligonucleotide that has 20 to 31 bases, which collectively form a sequence that is 100% complementary to a segment of the pre-mRNA transcribed from exon 53 of the human dystrophin gene”

82. Dr. Stein takes the position that Term 1 should be construed in its entirety, rather than by addressing Terms 1a, 1b, and 1c individually:

I understand that Sarepta proposes considering this phrase in its entirety, whereas NS proposes excerpting out three subparts within the phrase (“a base sequence,” “a target region,” and “exon 53 of the human dystrophin pre-mRNA”) and analyzing each separately. In my opinion, Sarepta’s proposal should be adopted because reading the phrase as a whole provides the appropriate context for those three subparts.

Ex. 37 ¶ 45. I do not agree with him.

83. As noted above, I understand the claim construction inquiry to begin with a focus on the words of the claim themselves, regardless of whether the term at issue spans a single word, a short phrase, or large swath of the claim. My analysis above and below follows this principle by paying due consideration to related limitations and surrounding claim language, even where those limitations or language is not proposed as part of the “term” for construction. One need not construe surrounding claim language to consider the context it provides.

84. In fact, what Dr. Stein describes as “providing the appropriate context” actually materially alters the claims. This alteration is evident when one compares the claim that would result from inputting Sarepta’s individual constructions for Terms 1a, 1b, and 1c with its collective construction for the entirety of Term 1:

Claim Language	Sarepta’s Individual Constructions	Sarepta’s Collective Construction
“antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA”	“antisense oligonucleotide of 20 to 31 bases comprising <u>a linear sequence of bases</u> that is 100% complementary to consecutive bases of <u>a segment of the pre-mRNA transcribed from exon 53 of the human dystrophin gene</u> ”	“antisense oligonucleotide that has 20 to 31 bases, which collectively form a sequence that is 100% complementary to a segment of the pre-mRNA transcribed from exon 53 of the human dystrophin gene”

85. Most notably, Sarepta’s collective construction eliminates the language “comprising.” This changes the claim from meaning that the “antisense oligonucleotide of 20 to 31 bases” **includes** a “base sequence,” to meaning that the “20 to 31 bases” **is** the “base sequence.” As discussed above, this conflicts with the plain meaning of “comprising a base sequence,” which, according to the specification, means “the inclusion of a stated integer [base sequence] . . . but not

the exclusion of any other integer or group of integers [another base sequence or bases sequences].”  
'851 Patent at 23:10-14.

86. Sarepta's collective construction also omits the “consecutive bases of” limitation. Even if “target region” merely means “a segment of the pre-mRNA,” as Sarepta proposes for Term 1b, omitting “consecutive bases of” materially changes how the “100% complementary” limitation operates. Instead of requiring complementarity to “**consecutive bases** of the target region” specifically, Sarepta's collective construction requires complementarity to the “target region” generally but has no requirement for that complementarity to be consecutive or linear. In fact, adding the word “collectively” while omitting “consecutive” introduces ambiguity, and would incorrectly suggest that the “base sequence” is complementary only in a collective manner, and not in a consecutive manner.

87. Thus, in my opinion, Sarepta's collective treatment of Terms 1a, 1b, and 1c does **not** provide “appropriate context,” as Dr. Stein opines. Ex. 37 ¶ 45. In fact, it does precisely the opposite. Sarepta's collective construction for Term 1 rewrites the claim in a manner that replaces or omits key language—such as “comprising” and “consecutive bases of”—and thus deprives the disputed terms of their original context.

88. For these reasons, and those provided in my analysis of Terms 1a, 1b, and 1c above, construing Terms 1a, 1b, and 1c individually most closely aligns with the plain language of the claims, and construing Term 1 collectively as Sarepta proposes would substantially depart from the claims' plain and ordinary meaning.

89. To the extent Term 1 is construed as a whole, however, I agree with NS's proposal. Because Terms 1b and 1c each leave a POSA without reasonable certainty regarding the scope of the claims, Term 1 as a whole would likewise leave a POSA without reasonable certainty. But, if

those terms were not indefinite, in my opinion, the appropriate construction for Term 1 would adopt NS's proposed construction for Term 1a, for the same reasons discussed above.

**XI. Term 2 – “wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)”**

90. The parties' positions for disputed Term 2 are shown below:

Claim Language	NS's Proposed Construction	Sarepta's Proposed Construction
“wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)”	<i>Indefinite</i>	“the target region is within nucleotides +23 to +69 of exon 53 of the human dystrophin pre-mRNA”

91. As highlighted below, using claim 1 of the '851 Patent as exemplary, Term 2 is the wherein clause found immediately after the language “consecutive bases of”:

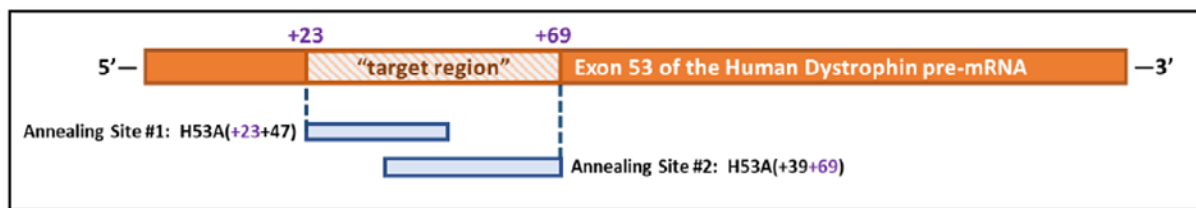
1. An antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA, **wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)**, wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, and wherein the antisense oligonucleotide induces exon 53 skipping; or a pharmaceutically acceptable salt thereof.

92. In my opinion, a person skilled in the art would have understood the defined “target region” to span nucleotides +39 to +47—the beginning marked by the start of annealing site H53A(+39+69), and the end marked by the end of annealing site H53(+23+47). Despite this understanding, however, it is my opinion that a person skilled in the art would still struggle to understand the scope of the claim with reasonable certainty because it is unclear how an “antisense oligonucleotide of 20 to 31 bases comprising . . . at least 12 consecutive bases . . .” could be “100% complementary to consecutive nucleotides” of a “target region” that is only nine bases long, *i.e.*,

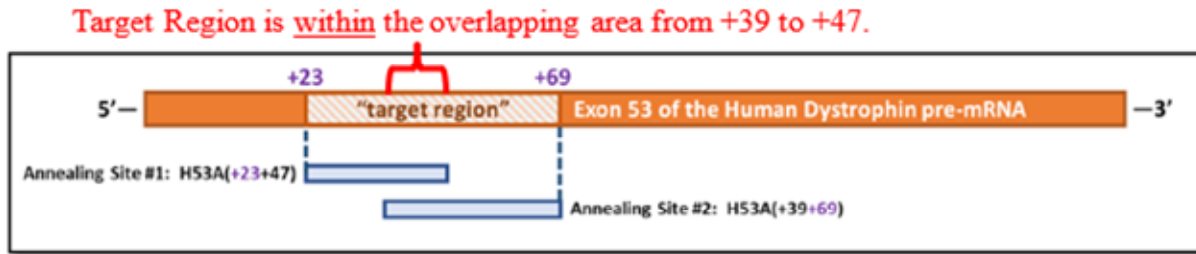
nucleotides +39 to +47. Thus, it is my opinion that the disputed phrase “wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)” is indefinite.

93. Sarepta and Dr. Stein argue that a person skilled in the art would understand the phrase to mean “the target region is within nucleotides +23 to +69 of exon 53 of the human dystrophin pre-mRNA.” I disagree. For one thing, the phrase requires the “target region” to be “within” *both* annealing sites, and Sarepta’s and Dr. Stein’s proposed construction includes nucleotides that are not (specifically, nucleotides +23 to +38 and +48 to +69). Additionally, it is unclear whether Sarepta’s proposed construction requires “the target region” to be “*within* nucleotides +23 to +69 of exon 53 of the human dystrophin pre-mRNA” or whether the “target region” is the *entire region* “spanning from nucleotides +23 to +69.” For example, Dr. Stein states that:

A skilled artisan would have understood that these **overlapping annealing sites define a target region** within exon 53 of the human dystrophin pre-mRNA. Specifically, as illustrated below, **these two annealing sites identify a region spanning from nucleotide +23 to nucleotide +69 of exon 53** of the human dystrophin pre-mRNA, the beginning marked by the first annealing site H53A(+23+47) and the end marked by the second annealing site H53A(+39+69).)



Ex. 37 ¶ 88; *id.* ¶ 89. I have annotated Dr. Stein’s drawing to show the correct interpretation of target region in the image below.



94. I have also reviewed the prosecution history of the UWA Patents cited by the parties (as listed in Exhibit B to the Joint Claim Construction Chart). In my opinion, the prosecution history confirms that a POSA would be uncertain regarding Term 2 and the scope of the claims.

95. Notably, in prosecuting the '827 Patent, the Office adopted the same meaning for Term 2 as I describe above—that “[t]he target region within the annealing sites H53A(+23+47) and H53A(+39+69) is 9 base long (i.e., 47-39)” —and rejected the claim as indefinite. Ex. 23 at SRPT-VYDS-0006254-55. In response, Applicant deleted Term 2 from the pending claims, *id.* at SRPT-VYDS-0006275, and argued for the same interpretation as Sarepta and Dr. Stein. *Id.* at SRPT-VYDS-0006276-77. However, because the claim was allowed with this deletion, *id.* at SRPT-VYDS-0006469-73, the Office never appears to have changed its position regarding the meaning of Term 2, or its indefiniteness.

## **XII. Term 3 – “in which uracil bases are thymine bases”**

96. The parties’ positions for disputed Term 3 are shown below:

Claim Language	NS’s Proposed Construction	Sarepta’s Proposed Construction
“in which uracil bases are thymine bases”	<i>Indefinite</i>	“the antisense oligonucleotide has thymine bases instead of uracil bases”

97. As highlighted below, using claim 1 of the '851 Patent as exemplary, Term 3 is found immediately after the recitation of SEQ ID NO: 195:

1. An antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA, wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69), wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195), **in which uracil bases are thymine bases**, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, and wherein the antisense oligonucleotide induces exon 53 skipping; or a pharmaceutically acceptable salt thereof.

98. In my opinion, a POSA would not be able to determine the scope of the phrase “in which uracil bases are thymine bases,” as that phrase is used in the UWA Patents, because a POSA would not be able to determine, with reasonable certainty, what portion of the claim the phrase “in which uracil bases are thymine bases” is supposed to modify. Accordingly, a POSA would determine that the claims of the UWA Patents are indefinite due to the inclusion of the phrase “in which uracil bases are thymine bases.”

99. In my opinion, there are at least two equally likely interpretations of claim scope due to the ambiguity in the claims’ use of the phrase “in which uracil bases are thymine bases.”

100. I have reviewed Dr. Stein’s Declaration and Sarepta’s Opening Brief, and agree that one of the potential interpretations is that the phrase “in which uracil bases are thymine bases” modifies the “antisense oligonucleotide” of the claims as a whole. In other words, one reasonable interpretation is that the use of the phrase “in which uracil bases are thymine bases” requires that no uracil bases may be used in the entire antisense oligonucleotide.

101. However, in my opinion, Dr. Stein and Sarepta have ignored a separate, and equally likely interpretation of the claims’ use of “in which uracil bases are thymine bases.” Under this interpretation, the phrase “in which uracil bases are thymine bases” modifies the language immediately preceding the phrase—namely that the uracil bases of the specifically recited SEQ ID NO: 195 are replaced with thymine bases in the claims.

102. In my opinion, the existence of these two, equally likely interpretations renders the claims of the UWA Patents indefinite, because a POSA, reading the claims in light of the specification and prosecution history, is unable to determine the scope of the claims with reasonable certainty.

103. Specifically, a POSA is unable to determine whether an antisense oligonucleotide, which includes at least 12 consecutive bases of SEQ ID NO: 195 (in which the uracil bases of the at least 12 consecutive bases of SEQ ID NO: 195 are thymine bases) will fall within the scope of the claims if the antisense oligonucleotide also includes uracil bases at other points within the molecule. For example, each of the below oligonucleotides has at least 12 consecutive bases of SEQ ID NO: 195 (in which the uracil bases of the 12 consecutive bases of SEQ ID NO: 195 are thymine bases) but contains uracil bases at other points in the molecule:

SEQ ID NO: 195	CUG AAG GUG UUC UUG UAC UUC AUC C
Example 1	<u>CTG AAG GTG TTC</u> UUG UAC UUC AU
Example 2	AAG GUG UUC <u>UTG TAC TTC ATC C</u>
Example 3	CCU CCG GUU <u>CTG AAG GTG TTC</u>
Example 4	<u>TG TAC TTC ATC CCA</u> CUG AUU C
Example 5	CUG <u>AAG GTG TTC TTG TAC</u> UUC AUC

\* The underlined bases are the at least 12 consecutive bases of SEQ ID NO: 195 (in which the uracil bases of the at least 12 consecutive bases of SEQ ID NO: 195 are thymine bases).

104. Each of the theoretical oligonucleotides listed above is an example only—and there are many others. In my opinion, due to the ambiguity of the phrase “in which uracil bases are thymine bases” used in the USA Patent claims, a POSA would be unable to determine with reasonable certainty if these examples—and similar other oligonucleotides—would fall within the scope of the claims. Accordingly, the claims of the UWA Patents are invalid.

**A. Oligonucleotides With Both Uracil and Thymine Bases Are Made and Studied**

105. As an initial matter, Dr. Stein and Sarepta may argue that this difference in claim scope is irrelevant. Indeed, Dr. Stein has argued that “[t]he use of uracil bases or thymine bases, but not both, is consistent with how antisense oligonucleotides were typically made in the art.” Ex. 37 ¶ 80; *see also id.* at 81-83.

106. I agree that in many cases, oligonucleotides use only thymine or uracil bases—not both. However, oligonucleotides using both thymine and uracil bases exist and have been studied.

107. Specifically, US Patent No. 8,084,601 (the “’601 Patent”) was filed on September 10, 2009 and issued on December 27, 2011. The ’601 Patent lists oligonucleotides having specific bases that can cause skipping in exon 53 of the DMD gene:

```

                                     (SEQ ID NO: 10)
j)   CXG XXG CCX CCG GXX CXG AAG GXG XXC XXG;

                                     (SEQ ID NO: 11)
k)   CAA CXG XXG CCX CCG GXX CXG AAG GXG XXC;
or

                                     (SEQ ID NO: 12)
l)   XXG CCX CCG GXX CXG AAG GXG XXC XXG XAC,
```

Ex. 38, ’601 Patent at 3:55-63.

108. The specification clarifies that the “base ‘X’ in the above base sequences is defined as being thymine (T) or uracil (U). . . . The base sequence of the molecule may contain all thymines, all uracils or a combination of the two.” *Id.* at 6:12-19.

109. Notably, each of the three sequences listed contains at least 12 consecutive bases that overlap with SEQ ID NO: 195 of the UWA Patents:

j) CXG XXG CCX CCG GXX CXG AAG GXG XXC XXG; (SEQ. ID NO.: 10)

k) CAA CXG XXG CCX CCG GXX CXG AAG GXG XXC; (SEQ. ID NO.: 11)  
or

l) XXG CCX CCG GXX CXG AAG GXG XXC XXG XAC; (SEQ. ID NO.: 12)

'601 Patent at 3:55-63 (annotations added showing overlap with SEQ ID NO: 195 of the UWA Patents). Thus, if a POSA replaced the “X” bases inside the red boxes with “Ts” and the “X” bases outside the red boxes with “U” bases—creating an oligonucleotide with a combination of thymines and uracils as is expressly contemplated by the '601 Patent—a POSA would be unable to determine if the oligonucleotide fell inside or outside the claims of the UWA Patents.

110. Further, a POSA would understand that PMO or morpholino oligonucleotides could also include a mixture of both thymine and uracil bases. Indeed, at the time of the priority date of the UWA Patents, a POSA would have known that PMOs could be made using uracils and thymines.

111. Specifically, a review article by James Summerton and Dwight Weller from 1997 describes the design and preparation of morpholinos, and notes that structure of morpholino oligomers includes uracil bases:

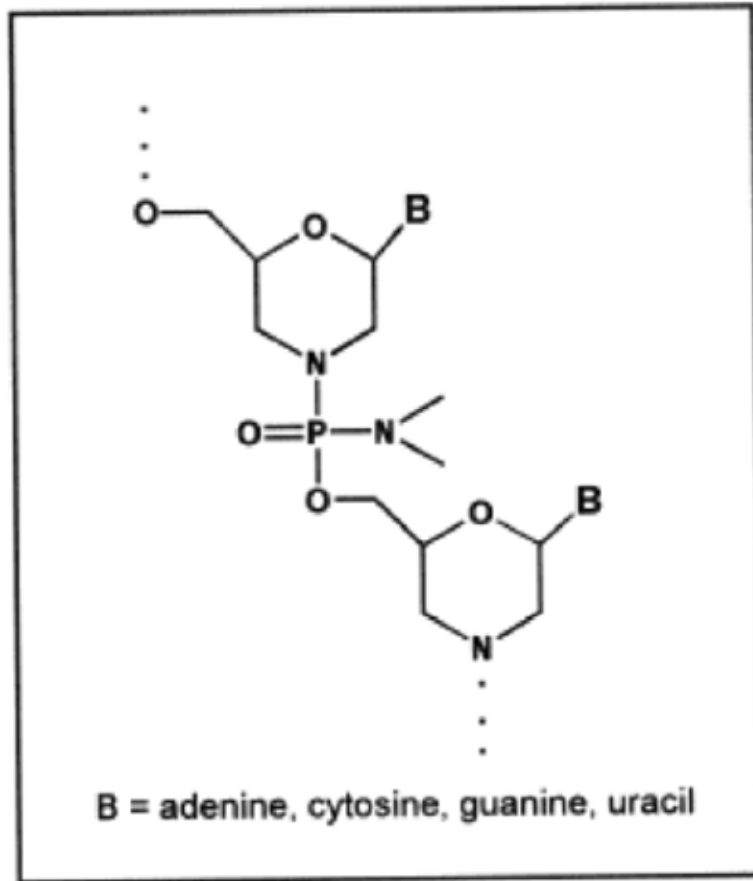


FIG. 2. Morpholino oligo structure.

112. Ex. 24 at 188. However, the same review article notes that oligonucleotides can also use thymine bases. *Id.* (“One then assembles an oligomer of genetic bases (adenine, cytosine, guanine, and thymine or uracil) complementary to that selected sequence.”).

113. Indeed, by at least 1998, a POSA would have understood that morpholinos could use both uracil and thymine bases, because, in “1998, Gene Tools, the sole commercial producer of research quantities of Morpholinos, switched from uracils to thymines in the production of Morpholinos.” Ex. 39, Gene Tools, LLC, Morpholino History, Production, and Properties, available at [https://www.gene-tools.com/history\\_production\\_and\\_properties](https://www.gene-tools.com/history_production_and_properties) (last accessed Feb. 6, 2023). Thus, before 1998, Gene Tools sold morpholino oligonucleotides having uracil bases, but

after 1998, Gene Tools sold morpholino oligonucleotides having thymine bases. Accordingly, by the priority date of the UWA Patents, a POSA would thus have been fully aware that morpholino oligonucleotides could contain uracil bases and thymine bases.

114. Moreover, while the '601 Patent suggests that when the sequences identified above (SEQ ID NO.: 10, 11, or 12) use PMOs, "X will be T as this base is used when producing PMOs," a POSA would have understood that the '601 Patent was specifically referring to PMOs made by Gene Tools "the sole commercial producer of research quantities of Morpholinos." *See* Ex. 38, '601 Patent at 10:33-35 ("All AOs were synthesized as phosphorodiamidate morpholino oligos (PMOs) by Gene Tools LLC (Philomath Oreg., USA)."). A POSA would have known that PMOs could also be produced using uracil bases. Ex. 24 (describing the preparation of morpholinos using uracil bases); Ex. 39 (confirming that before 1998, Gene Tools made morpholinos using uracil bases). Accordingly, a POSA would have understood that if they synthesized their own morpholinos, rather than purchasing morpholinos from Gene Tools, a POSA could make morpholinos with both uracil and thymine bases.

115. A POSA would also have known that the binding efficacy of an oligonucleotide can be improved by optimizing each individual base—including by optimizing whether a specific location is a uracil or thymine. *See* '851 Patent at 27:60-64; *see also* Ex. 40 at 7 (describing the advantages of using uracil and thymine in steric blocking antisense oligonucleotides). A POSA would have understood that an oligonucleotide used for treating patients should be optimized so as to provide the greatest efficacy. Indeed, at least one oligonucleotide medication currently approved by the FDA includes both uracil bases and a thymine base. *See* Ex. 41 at 5 (describing the structure of LEQVIO—a type of oligonucleotide that is a double stranded RNA called

siRNA—that includes dT (i.e., thymidine—a deoxynucleoside with a *thymine* base) and Um (uracil 2'-OMe ribonucleotide—a nucleotide with a uracil base).

116. Given that oligonucleotides containing both uracil and thymine bases are known and discussed by various sources, the ambiguity in claim scope of the UWA Patents is relevant and practically meaningful.

**B. The Claim Language Itself Supports Both Interpretations**

117. In my opinion, the claim language of the UWA Patents supports both interpretations of the phrase “in which uracil bases are thymine bases.”

118. Dr. Stein argues that the claim language “makes it clear that it modifies the claimed “antisense oligonucleotide” as a whole, not just some unspecified portion of it.” Ex. 37 ¶ 72.

119. In his analysis, Dr. Stein spaces out and annotates Claim 1 of the '590 Patent as follows:

1. An antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA,

**wherein** the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195),

**in which** uracil bases are thymine bases,

**wherein** the antisense oligonucleotide is a morpholino antisense oligonucleotide, **and**

**wherein** the antisense oligonucleotide induces exon 53 skipping;

or a pharmaceutically acceptable salt thereof.

Ex. 37 ¶ 72.

120. Dr. Stein argues that the claim “describes four characteristics of the claimed antisense oligonucleotides, each identified by a ‘wherein’ or ‘in which’ clause,” such that the phrase “in which uracil bases are thymine bases” must modify the “claimed antisense oligonucleotide” as a whole. Ex. 37 ¶ 73.

121. I agree with Dr. Stein that this is one reasonable way a POSA could understand and break down the claim language itself. However, in my opinion, the claim language provides slightly stronger support for a different interpretation.

122. First, I note that the actual text of Claim 1 of the ’590 Patent is not spaced out or annotated in the manner that Dr. Stein provides. Instead, Claim 1 is simply one long sentence with no spacing of the different clauses.

What is claimed is:  
1. An antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA, wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, and wherein the antisense oligonucleotide induces exon 53 skipping; or a pharmaceutically acceptable salt thereof.

Ex. 2, ’590 Patent at Claim 1.

123. In my opinion, a POSA would also understand that Claim 1 of the ’590 Patent could be written and annotated as follows:

1. An antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA,  
**wherein the base sequence** comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195), **in which** uracil bases are thymine bases,

**wherein the antisense oligonucleotide** is a morpholino antisense oligonucleotide, and

**wherein the antisense oligonucleotide** induces exon 53 skipping;

or a pharmaceutically acceptable salt thereof.

'590 Patent at Claim 1 (annotations added).

124. These annotations demonstrate how a POSA would reasonably understand the phrase “in which uracil bases are thymine bases” to modify the listing of SEQ ID NO: 195 that immediately precedes the phrase, rather than the “antisense oligonucleotide” as a whole.

125. Specifically, unlike each of the other clauses in the claim, which use “wherein,” the relevant “uracil bases are thymine bases” uses the beginning preposition “in which.” This suggests that it is different from the “wherein” clauses and should be included with the previous language—instead of set apart by itself. This organization of the claim is further supported by the fact that each of the “wherein” clauses specifically identifies a subject that it modifies—either “the base sequence” or “the antisense oligonucleotide.” The “in which” clause does not specify that it modifies either “the base sequence” or “the antisense oligonucleotide,” further setting it apart from the “wherein” clauses. Accordingly, it is at least equally likely, if not more likely, that the claim language and structure as a whole supports an understanding that the phrase “in which uracil bases are thymine bases” should be interpreted to modify the listing of SEQ ID NO: 195 that immediately precedes the phrase, rather than the “antisense oligonucleotide” as a whole.

126. The specific language of the phrase “in which uracil bases are thymine bases” also supports an interpretation that the phrase modifies the listing of SEQ ID NO: 195 rather than the “antisense oligonucleotide” as a whole. By using the language “uracil bases are thymine bases,” the inventor is informing the public that a specific pre-existing series of bases that already includes “uracil bases” should be **changed** to include thymine bases instead. Such language fits more neatly

if it is interpreted to refer to the listing of SEQ ID NO: 195 rather than the “antisense oligonucleotide” as a whole. Antisense oligonucleotides are made with specific bases—a POSA would not make an antisense oligonucleotide with particular bases and then go back and reconstruct it with different bases. Instead, a POSA would just make the antisense oligonucleotide with the bases they desired from the beginning.

127. Accordingly, if the “in which” phrase was intended to refer to the claimed antisense oligonucleotide as a whole, more appropriate language would have been “in which the antisense oligonucleotide does not include uracil bases” or “in which the antisense oligonucleotide contains thymine bases instead of uracil bases.”

128. However, the language “uracil bases are thymine bases” is appropriate if it is interpreted to modify the listing of SEQ ID NO: 195, because SEQ ID NO: 195 specifically lists a series of bases that includes uracil bases (CUG AAG GUG UUC UUG UAC UUC AUC C). Thus, while the claim language of the phrase “in which uracil bases are thymine bases,” is ambiguous at best, it more strongly supports an interpretation that the phrase “in which uracil bases are thymine bases” modifies the listing of SEQ ID NO: 195 rather than the “antisense oligonucleotide” as a whole.

**C. The Specification of the UWA Patents Does Not Assist A POSA In Determining Claim Scope**

129. In my opinion, the specification of the UWA Patents does not remedy or clarify the ambiguity in claim interpretation caused by the use of the phrase “in which uracil bases are thymine bases.”

130. Dr. Stein and Sarepta have argued that “a skilled artisan would have understood the thymine bases phrase to mean that the claimed antisense oligonucleotide, which is a *morpholino* antisense oligonucleotide, has thymine bases instead of uracil bases.” Ex. 37 ¶¶ 74-75.

Specifically, Dr. Stein has pointed to examples in Table 1A of the UWA Patents, which includes *exemplary* antisense oligonucleotides:

TABLE 1A		
Description of 2'-O-methyl phosphorothioate antisense oligonucleotides that have been used to date to study induced exon skipping during the processing of the dystrophin pre-mRNA. Since these 2'-O-methyl antisense oligonucleotides are more RNA- like, U represents uracil. With other antisense chemistries such as peptide nucleic acids or morpholinos, these U bases may be shown as "T".		
SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5'-3')
1	H8A(-06+18)	GAU AGG UGG UAU CAA CAU CUG UAA
2	H8A (-03+18)	GAU AGG UGG UAU CAA CAU CUG
3	H8A(-07+18)	GAU AGG UGG UAU CAA CAU CUG UAA G
4	H8A(-06+14)	GGU GGU AUC AAC AUC UGU AA
5	H8A(-10+10)	GUA UCA ACA UCU GUA AGC AC

*Id.*

131. Dr. Stein concludes that, since (i) these examples contain uracil bases, not a mixture of uracil and thymine bases, and (ii) Table 1A “guides a skilled artisan to substitute thymine bases for those uracil bases . . . not a combination of uracil bases and thymine bases,” then (iii) a POSA would have understood that the claimed antisense oligonucleotides should include only thymine bases, not a mixture. *Id.* I disagree with Dr. Stein’s conclusions.

132. First, I note that Table 1A includes only examples of potential oligonucleotides, as Dr. Stein admits. *Id.* Such examples are not representative of scope of the oligonucleotides claimed by the UWA Patents.

133. Second, I note that the legend of Table 1A states that, with “morpholinos, these [uracil] bases may be shown as [thymine.]” ’851 Patent at Table 1A. In other words, the ’851 Patent teaches that, when making morpholino antisense oligomers, the uracil bases may be

modified or substituted with thymine bases. *Id.* At no point does the '851 Patent teach or suggest that such a modification or substitution is required for all uracil bases in a morpholino antisense oligomer.

134. My understanding is consistent with other portions of the specification of the UWA Patents. Specifically, the UWA Patents teach that “[o]ligonucleotides may also include nucleobase (often referred to in the art simply as ‘base’) modifications or substitutions.” ’851 Patent at 27:35-37. One of the reasons identified for such nucleobase modifications is that “[c]ertain nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention.” *Id.* at 27:37-39. In other words, substituting or modifying specific nucleobases in an oligonucleotide may help the oligonucleotide bind more efficiently to specific locations of mRNA. As shown by Table 1A, one such potential substitution or modification is replacing certain uracil bases with thymine bases.

135. Third, the specifications of the UWA Patents expressly teach that “[i]t is not necessary [for] all positions in a given compound to be uniformly modified, and . . . the aforementioned modifications [including nucleobase modifications or substitutions] may be incorporated . . . at a single nucleoside<sup>7</sup> within an oligonucleotide.” *Id.* at 27:60-64. In other words, the UWA Patents teach that when making substitutions or modifications—including nucleobase substitutions or modifications—such substitutions or modifications can be performed on a nucleotide-by-nucleotide basis, such that an entire oligonucleotide does not need to include the same substitutions or modifications.

136. Thus, a POSA, when viewing the specification of the UWA Patents as a whole would understand that, (i) the nucleobases of morpholino antisense oligonucleotides could be

---

<sup>7</sup> A nucleoside is essentially a nucleotide without the phosphate linkage.

modified such that the antisense oligonucleotides included thymine bases rather than uracil bases, and (ii) such modifications could be made on specific portions of a morpholino antisense oligonucleotide, such that the antisense oligonucleotide would include both thymine bases and uracil bases as part of the molecule.

137. Accordingly, the teachings of the specifications of the UWA Patents would not provide a POSA with reasonable certainty as to the meaning of the phrase “in which uracil bases are thymine bases.”

**D. The Prosecution History Does Not Resolve the Ambiguity**

138. Dr. Stein cites to one Office Action in the prosecution history of a related patent application in order to support his interpretation that the phrase “in which uracil bases are thymine bases” modifies the oligonucleotide as a whole. Ex. 37 ¶¶ 76-79. I disagree with Dr. Stein’s conclusions. In my opinion, the prosecution history identified by Dr. Stein does not resolve the ambiguity surrounding the correct interpretation of the phrase “in which uracil bases are thymine bases.”

139. First, Dr. Stein references two parts of the same Office Action—an argument written by the patent applicant—in support of his opinions:

Specifically, the pending claims are drawn to an antisense oligonucleotide having the following elements: (i) 25 bases comprising a base sequence that is 100% complementary to 25 consecutive bases of exon 53 of the human dystrophin pre-mRNA; (ii) 20 consecutive bases of SEQ ID NO: 193; (iii) uracil bases are thymine bases; (iv) the antisense oligonucleotide is a morpholino; (v) the antisense oligonucleotide induces exon 53 skipping; *and* (vi) the antisense oligonucleotide is chemically linked to a polyethylene glycol chain.

Ex. 20 at SRPT-VYDS-0094179.

Further, none of the cited references teach or suggest combining the elements to result in the claimed antisense oligonucleotide. Specifically, there is no teaching or suggestion to generate an antisense oligonucleotide of 25 bases, wherein the antisense oligonucleotide comprises 20 consecutive bases of SEQ ID NO: 193, and wherein uracil bases are thymine bases, and wherein the antisense oligonucleotide is a morpholino, and wherein the resulting antisense oligonucleotide induces exon 53 skipping of the human dystrophin pre-mRNA.

*Id.* at SRPT-VYDS-0094181.

140. Dr. Stein argues that “these statements from the applicant confirm that the thymine bases clause should be read to modify the claimed ‘antisense oligonucleotide.’” Ex. 37 ¶ 79. I disagree.

141. First, nothing in the Office Action discusses or evaluates the two potential interpretations of the phrase “in which uracil bases are thymine bases.” *See* Ex. 20 at SRPT-VYDS-0094177 – 85. Instead, the applicants’ argument was focused on whether the prior art disclosed substituting uracil bases with thymine bases at all—and did not focus on the specific location of the substitution. *Id.* at SRPT-VYDS-0094180 (“Applicants wish to point out that there is absolutely nothing in [the prior art reference] about substituting uracil bases . . . with thymine bases. In fact the word ‘thymine’ (or its structure) is not described anywhere.”). A POSA would have understood that the office action’s listing of the various elements was included simply to describe all of the elements of the claim, and was not meant to clarify whether the phrase “in which uracil bases are thymine bases” should be applied to the “antisense oligonucleotide” as a whole, or to specific sequence listed immediately prior to the clause. For this reason, a POSA would not have placed much significance on the exact language of the listing and would instead have focused on the language of the claim itself.

142. Second, my opinion is further supported by the fact that the listings themselves are grammatically inconsistent. According to grammatical principles, the items in a list should have a parallel structure or grammatical form. *See* Ex. 42, *Parallel Structure*, The Writing Center,

George Mason University, available at <https://writingcenter.gmu.edu/writing-resources/grammar-style/parallel-structure> (last accessed Feb. 6, 2023). Here, the lists in the office action do not use a parallel structure for the lists. Instead, certain of the items specifically refer to the “antisense oligonucleotide” as the subject of the item (underlined in red, below). However, the uracil bases item includes a different structure (underlined in blue, below).

Specifically, the pending claims are drawn to an antisense oligonucleotide having the following elements: (i) 25 bases comprising a base sequence that is 100% complementary to 25 consecutive bases of exon 53 of the human dystrophin pre-mRNA; (ii) 20 consecutive bases of SEQ ID NO: 193; (iii) uracil bases are thymine bases; (iv) the antisense oligonucleotide is a morpholino; (v) the antisense oligonucleotide induces exon 53 skipping; **and** (vi) the antisense oligonucleotide is chemically linked to a polyethylene glycol chain.

Ex. 20 at SRPT-VYDS-0094179 (annotations added).

Further, none of the cited references teach or suggest combining the elements to result in the claimed antisense oligonucleotide. Specifically, there is no teaching or suggestion to generate an antisense oligonucleotide of 25 bases, wherein the antisense oligonucleotide comprises 20 consecutive bases of SEQ ID NO: 193, **and** wherein uracil bases are thymine bases, **and** wherein the antisense oligonucleotide is a morpholino, **and** wherein the resulting antisense oligonucleotide induces exon 53 skipping of the human dystrophin pre-mRNA.

*Id.* at SRPT-VYDS-0094181 (annotations added).

143. Given the inconsistent grammatical structure of these lists, it is difficult to ascribe any clear meaning. Specifically, because the phrase “uracil bases are thymine bases” is grammatically different than many of the other phrases, it is difficult to determine whether the applicant actually intended the phrase to modify the “antisense oligonucleotide” as a whole, or whether the phrase was intended to modify “SEQ ID NO: 193” that immediately precedes the phrase in both lists. Accordingly, in my opinion, the prosecution history fails to resolve the ambiguity related to the proper interpretation of the phrase “in which uracil bases are thymine bases” as used in the UWA Patents.

144. Moreover, while the phrase “in which uracil bases are thymine bases,” is ambiguous at best, even if the prosecution history slightly more strongly supports an interpretation that the phrase “in which uracil bases are thymine bases” modifies the “antisense oligonucleotide” as a whole, it is balanced by the language of the claim itself, which more strongly supports an interpretation that the phrase modifies the listing of SEQ ID NO: 195. Accordingly, the phrase “in which uracil bases are thymine bases” is indefinite when considering the intrinsic evidence as a whole.

**XIII. CONCLUSION**

145. I declare under penalty of perjury under the laws of the United States that the foregoing is true and correct. Executed at North Chicago, Illinois this 6th day of February, 2023.

A handwritten signature in black ink, reading "Michelle L. Hastings", written in a cursive style.

---

Michelle L. Hastings, Ph.D

# Exhibit 44

# Merriam-Webster



-



Hello,

Username






[Log In](#) [Sign Up](#)

- [Username](#)
  - [My Words](#)
  - [Recents](#)
  - [Settings](#)
  - [Log Out](#)
- [Games & Quizzes](#)
- [Thesaurus](#)
- [Features](#)
- [Word of the Day](#)
- [Shop](#)
- [Join MWU](#)
- [More](#)
  - [Shop M-W Books](#) ↗
  - [Join MWU](#) ↗
    - [Log In](#)
    - [Username](#)
    - [My Words](#)
    - [Recents](#)
    - [Account](#)
    - [Log Out](#)
- [Settings](#)
- [My Words](#)
- [Recents](#)
- [Account](#)
- [Log Out](#)


Est. 1828



[Dictionary](#) Definition

- [adverb](#) 
- [preposition](#) 
- [noun](#) 
- [adjective](#) 
- [adverb 4](#) 
  - [adverb](#)
  - [preposition](#)
  - [noun](#)
  - [adjective](#)
- 



- [Synonyms](#)
- [Example Sentences](#)
- [Word History](#)
- [Phrases Containing](#)
- [Entries Near](#)
- - [Cite this Entry](#)
  - [Share](#)
  - [Kids Definition](#)
  - [More from M-W](#)
- [Show more](#)
- [Show more](#)
  - [Citation](#)
  - [Share](#)
  - [Kids](#)
  - [More from M-W](#)
- Save Word 

  - To save this word, you'll need to log in.  
[Log In](#)



# within

1 of 4

## adverb

with·in [wi- 'thin](#) - 'thin

[Synonyms of within](#)

1

: in or into the interior : [inside](#)

2

: in one's inner thought, disposition, or character : [inwardly](#)

search within for a creative impulse—Kingman Brewster †1988

within

2 of 4



preposition

- 1  
→  
—used as a function word to indicate enclosure or containment
- 2  
→  
—used as a function word to indicate situation or circumstance in the limits or compass of: such as  
a  
: before the end of  
gone within a week  
b(1)  
: not beyond the quantity, degree, or limitations of  
live within your income  
(2)  
: in or into the scope or sphere of  
within the jurisdiction of the state  
(3)  
: in or into the range of  
within reach  
within sight  
(4)  
→  
—used as a function word to indicate a specified difference or margin  
came within two points of a perfect markwithin a mile of the town
- 3  
: to the inside of : [into](#)  
sunk the sea within the earth—William Shakespeare

From  
O'Hare

One-way as low as\*

\$59

\*First travel date available  
2/28/2023 with 18 travel days in  
schedule. Seats/days/mkts lmted;  
restr./excl. apply.

Southwest

Book now



within

3 of 4

noun

- : an inner place or area  
revolt from within
- within
- 4 of 4



## adjective

: being inside : [enclosed](#)  
the within indictment



## Synonyms

Noun

- [innards](#)
- [inside](#)
- [interior](#)

[See all Synonyms & Antonyms in Thesaurus](#) >

## Example Sentences

Adverb We could hear sounds coming from *within*. The sign on the door says “Help Wanted: Inquire *Within*.” We all try to appear strong and attempt to hide the scared little child *within*. [See More](#)

Recent Examples on the Web

Adverb

There were 636 delays into, *within* or out of the U.S. as of Thursday morning, according to flight-tracking website FlightAware. —David Koenig, *Anchorage Daily News*, 12 Jan. 2023 In his story for T, writer at large Adam Bradley visits with the artists making work about (and sometimes *within*) the American carceral system. —Hanya Yanagihara, *New York Times*, 18 Aug. 2022 [See More](#)

These example sentences are selected automatically from various online news sources to reflect current usage of the word 'within.' Views expressed in the examples do not represent the opinion of Merriam-Webster or its editors. [Send us feedback](#).



## Word History

### Etymology

#### Adverb

Middle English *withinne*, from Old English *withinnan*, from *with* + *innan* inwardly, within, from *in*

#### First Known Use

#### Adverb

before the 12th century, in the meaning defined at [sense 1](#)

#### Preposition

12th century, in the meaning defined at [sense 1](#)

#### Noun

15th century, in the meaning defined [above](#)

#### Adjective

1748, in the meaning defined [above](#)

#### Time Traveler

The first known use of *within* was before the 12th century

[See more words from the same century](#)

## Phrases Containing *within*

- [in/within the space of](#)
- [wheels within wheels](#)
- [within an ace of](#)
- [live within/beyond one's means](#)
- [within an inch of](#)
- [in/within living memory](#)

- [come in/within sight of \(something\)](#)
- [within easy reach](#)
- [within/in walking distance](#)

[See More](#)

- [in/within the space of](#)
- [wheels within wheels](#)
- [within an ace of](#)
- [live within/beyond one's means](#)
- [within an inch of](#)
- [in/within living memory](#)

[See More](#)

## Dictionary Entries Near *within*

[with hopes of](#)

within

[within a whisker of](#)

## Cite this Entry

Style

MLA ▼

“Within.” *Merriam-Webster.com Dictionary*, Merriam-Webster, <https://www.merriam-webster.com/dictionary/within>. Accessed 4 Feb. 2023.

 [Copy Citation](#)

## Share

[Post the Definition of within to Facebook](#)



[Facebook](#)

[Share the Definition of within on Twitter](#)



[Twitter](#)

## Kids Definition

within

1 of 3 adverb

with·in [with- 'in](#) [with-](#)

1

: in or into the interior : [inside](#)

2

: inside oneself : [inwardly](#)

look *within* for imaginative ideas

within

2 of 3 preposition

1

: in or into the inner part of

*within* the house

2

: not beyond the bounds or limits of

*within* sight

within

3 of 3 noun

: an inner place or area



## More from Merriam-Webster on *within*

Nglish: [Translation of \*within\* for Spanish Speakers](#)

Britannica English: [Translation of \*within\* for Arabic Speakers](#)


Last Updated: 2 Feb 2023 - Updated example sentences



## Love words? Need even more definitions?

Subscribe to America's largest dictionary and get thousands more definitions and advanced search—ad free!

MERRIAM-WEBSTER UNABRIDGED



Plan Your Next Event Today  
— Our 30 Hotel Brands Are  
Ready to Bring You Together  
Again.

Marriott Bonvoy



WORD OF THE DAY

scrutinize 

[See Definitions and Examples »](#)

Get Word of the Day daily email!

Your email address

**SUBSCRIBE**

**know about APRETUDE?**

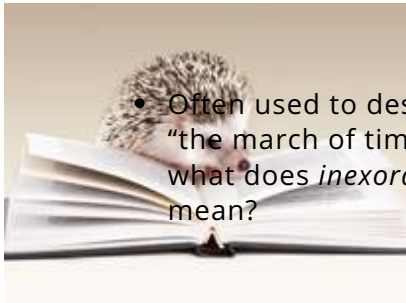
Before receiving APRETUDE to reduce your risk of getting HIV-1, you must be HIV-1 negative to start APRETUDE. Do not receive APRETUDE unless you are tested and confirmed to be HIV-1 negative.

- Some HIV-1 tests can miss HIV-1 infection in a person who has recently become infected. Tell your healthcare provider if you had a flu-like illness within



Test Your Vocabulary

Challenging Words You Should Know



- Often used to describe “the march of time,” what does *inexorable* mean?

Unpredictable

Swift

Relentless

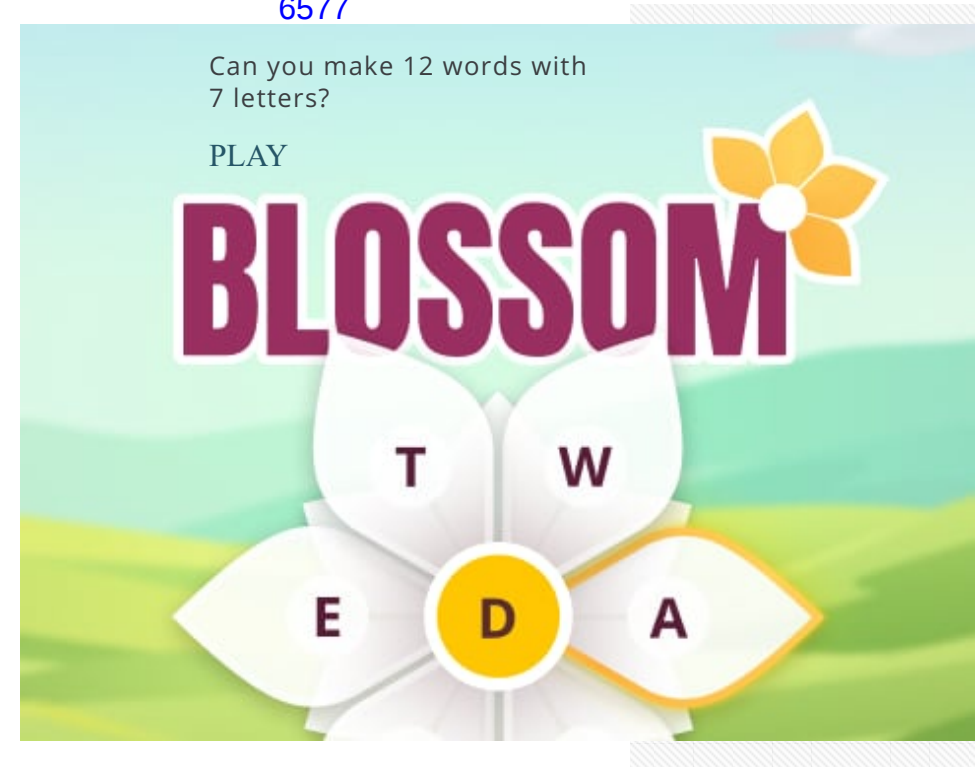
Slow



Test your knowledge - and maybe learn something along the way.

TAKE THE QUIZ





WORDS AT PLAY



## 14 Words Inspired by Dogs

- A lexicographer's best friend

## Great Big List of Beautiful and Useless Words, Vol. 4

More words, more beautiful, more

- useless

## 'Gray' vs. 'Grey': What is the Difference?

- Spelling isn't all black and white.

## When Were Words First Used?

- Look up any year to find out

### ASK THE EDITORS

---

## Weird Plurals

One goose, two geese. One moose,

- two... moose. Wh...



## Irregardless

It is in fact a real word (but that  
• doesn't mean ...

## Bring vs. Take

Both words imply motion, but the  
• difference may b...

## Defenestration

The fascinating story behind many  
• people's favori...

## WORD GAMES

---

### A Good Old-Fashioned Quiz

Can you name these antiquated  
items?

- [TAKE THE QUIZ >](#)

### Which Came First?

"Leggings" or "mom jeans"?  
"Chillax" or "dumpster..."

- [TAKE THE QUIZ >](#)



## True or False?

Test your knowledge - and maybe  
learn something a...

- [TAKE THE QUIZ >](#)

## Spelling Bee Quiz

Can you outdo past winners of the  
National Spelli...

- [TAKE THE QUIZ >](#)

Learn a new word every day. Delivered to your inbox!

Your email address



### OTHER MERRIAM-WEBSTER DICTIONARIES

- MERRIAM-WEBSTER'S UNABRIDGED DICTIONARY
- SCRABBLE® WORD FINDER
- MERRIAM-WEBSTER DICTIONARY API
- ENGLISH - SPANISH-ENGLISH TRANSLATION
- BRITANNICA ENGLISH - ARABIC TRANSLATION

FOLLOW US



Browse the Dictionary: A B C D E F G H I J K L M N O P Q R S T U  
V W X Y Z 0-9 BIO GEO

Home | Help | About Us | Shop | Advertising Info | Dictionary API | Contact Us |  
Join MWU | Videos | Word of the Year | Kid's Dictionary | Law Dictionary |  
Medical Dictionary | Privacy Policy | Terms of Use

Browse the Thesaurus | Browse the Medical Dictionary |  
Browse the Legal Dictionary | Browse the Kid's Dictionary

© 2023 Merriam-Webster, Incorporated

Information from your device can be used to personalize your ad experience.

[Do not sell or share my personal information.](#)



# Exhibit 45



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE  
United States Patent and Trademark Office  
Address: COMMISSIONER FOR PATENTS  
P.O. Box 1450  
Alexandria, Virginia 22313-1450  
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/645,842	07/10/2017	Stephen Donald WILTON	4140.015REIC	8351

153767 7590 07/12/2018  
STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.  
1100 NEW YORK AVENUE, N.W.  
WASHINGTON, DC 20005

EXAMINER
----------

PONNALURI, PADMASHRI

ART UNIT	PAPER NUMBER
----------	--------------

3991

NOTIFICATION DATE	DELIVERY MODE
-------------------	---------------

07/12/2018

ELECTRONIC

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

jcovert@sternekessler.com  
e-office@sternekessler.com

**Office Action Summary**Application No.  
15/645,842Applicant(s)  
WILTON ET AL.Examiner  
PADMASHRI PONNALURIArt Unit  
3991AIA (First Inventor to File)  
Status  
No**-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --****Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**Status**

- 1) ☒ Responsive to communication(s) filed on 7/10/17.  
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on \_\_\_\_.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on \_\_\_\_; the restriction requirement and election have been incorporated into this action.
- 4) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

**Disposition of Claims\***

- 5) ☒ Claim(s) 44-64 is/are pending in the application.  
5a) Of the above claim(s) \_\_\_\_ is/are withdrawn from consideration.
- 6) ☐ Claim(s) \_\_\_\_ is/are allowed.
- 7) ☒ Claim(s) 44-64 is/are rejected.
- 8) ☐ Claim(s) \_\_\_\_ is/are objected to.
- 9) ☐ Claim(s) \_\_\_\_ are subject to restriction and/or election requirement.

\* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see [http://www.uspto.gov/patents/init\\_events/pph/index.jsp](http://www.uspto.gov/patents/init_events/pph/index.jsp) or send an inquiry to [PPHfeedback@uspto.gov](mailto:PPHfeedback@uspto.gov).

**Application Papers**

- 10) ☐ The specification is objected to by the Examiner.
- 11) ☐ The drawing(s) filed on \_\_\_\_ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.  
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).  
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

**Priority under 35 U.S.C. § 119**

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

**Certified copies:**

- a) ☐ All b) ☐ Some\*\* c) ☐ None of the:
1. ☐ Certified copies of the priority documents have been received.
  2. ☐ Certified copies of the priority documents have been received in Application No. \_\_\_\_.
  3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

\*\* See the attached detailed Office action for a list of the certified copies not received.

**Attachment(s)**

- 1) ☒ Notice of References Cited (PTO-892)
- 2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b)  
Paper No(s)/Mail Date 2/7/18; 10/23/17; 10/18/17.
- 3) ☐ Interview Summary (PTO-413)  
Paper No(s)/Mail Date. \_\_\_\_.
- 4) ☐ Other: \_\_\_\_.

Application/Control Number: 15/645,842  
Art Unit: 3991

Page 2

The present application is being examined under the pre-AIA first to invent provisions.

### ***Procedural Posture***

This is a reissue application (filed 7/10/17) of US patent 8,455,636, Issued on June 4, 2013.

*For reissue applications filed on or after September 16, 2012, all references to 35 U.S.C. 251 and 37 CFR 1.172, 1.175, and 3.73 are to the current provisions.*

### ***Priority***

US patent 8,455,636 is issued from US application 13/271,080, filed on Oct. 11, 2011; Which is a continuation of application 12/837,359, filed on Jul 15, 2010, now Pat. No. 8,232,384, which is a continuation of 11/570,691, filed as PCT/AU2005/000943 on Jun. 28, 2005, now Pat. No. 7,807,816.

### ***Ongoing Duty to Disclose***

Applicant is reminded of the continuing obligation under 37 CFR 1.178(b), to timely apprise the Office of any prior or concurrent proceeding in which Patent No. 8,455,636 is or was involved. These proceedings would include interferences, reissues, reexaminations, and litigation.

Applicant is further reminded of the continuing obligation under 37 CFR 1.56, to timely appraise the Office of any information which is material to patentability of the claims under consideration in this reissue application.

These obligations rest with each individual associated with the filing and prosecution of this application for reissue. See also MPEP §§ 1404, 1442.01 and 1442.04.

### ***PTAB and CAFC Decisions***

Patent Trial and Appeal Board ordered that claims 1-43 (all claims) of US Patent 8,455,636 be cancelled (35 USC. 135(a)(2010) (See PTAB interference No. 106,007 for the decision mailed on April 29, 2016).

The appeal to the CAFC was dismissed on 7/27/17 (see the CAFC decision mailed on 7/27/17).

### ***Status of Claims***

Claims 1-43 were originally present in the issued US patent 8,455,636.

In this reissue application new claim 44 was added by the amendment filed on 7/10/17.

Application/Control Number: 15/645,842  
Art Unit: 3991

Page 3

Original claims 1-43 are canceled 12/15/17 amendment in this reissue application.

Claim 44 was amended, and new claims 45-64 are added by the amendment filed on 1/29/18.

Claims 44-64 are currently pending in this reissue application.

### *Claim Amendment*

The amendment filed 1/29/18 proposes amendments by adding new claims 44-64 that do not comply with 37 CFR 1.173(b) and (c), which sets forth the manner of making amendments in reissue applications. A supplemental paper correctly amending the reissue application is required as a response to this office action.

New claims recite an injectable solution comprising an antisense oligonucleotide of 25 bases and comprises at least 20 consecutive bases of sequence of SEQ ID NO: 193. The antisense oligonucleotide is 100 % complementary to 25 consecutive nucleotides of target region of exon 53 of the human dystrophin pre-mRNA, and the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69). The specification in Table 1A discloses the antisense oligonucleotides that have been used to study induced exon skipping. Table 1 A lists antisense oligonucleotides of SEQ IDNO: 193 which is complimentary to the annealing site H53A(+39+69) and antisense oligonucleotide of SEQ IDNO: 195 which is complimentary to the H53A(+23+47). However, the specification nowhere discloses "target region is **within** annealing site H53A(+23+47) and annealing site H53A(+39+69)" as in the present new independent claims 44, 46, 47, 49, 50, 52, 53, 55, 56, 58, 59, 61, 62 and 64. Applicants failed to include an explanation of the support in the disclosure of the patent for the new claim limitations. See 37 CFR 1.173 (c).

### *Reissue Declaration*

1. The reissue oath/declaration filed 12/12/17 with this application is defective because the error which is relied upon to support the reissue application is not an error upon which a reissue can be based. See 37 CFR 1.175 and MPEP § 1414.

Application/Control Number: 15/645,842

Page 4

Art Unit: 3991

This is a broadening reissue application, since the original claims in the `636 patent did not cover an “an injectable solution” comprising an antisense oligo nucleotide, and the injectable solution is “formulated for” different administration routes and further that the target region is “within” the two annealing sites H53A(+23+47) and H53A(+39+69) as recited in the new claims. Thus, the new claims in this reissue enlarge the scope of the patent claims. See MPEP 1412.03.

A. Claims 44-64 are rejected under 35 U.S.C. 251 as being broadened in a reissue application filed outside the two year statutory period. The present reissue application is filed more than 2 years after the issue date of US Patent 8,455,636. The newly added claims recite “an injectable solution comprising an antisense oligonucleotide;” and “the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69);” and wherein the injectable solution is “formulated for” different routes of administration (i.e., parenteral, intravenous, intramuscular, subcutaneous, intraocular, oral or transdermal administration). The scope of the original claim “isolated antisense oligonucleotide” has been broadened by changing the annealing site of the antisense molecule. Therefore, this is a broadening Reissue application. A claim is broader in scope than the original claims if it contains within its scope any conceivable product or process which would not have infringed the original patent. A claim is broadened if it is broader in any one respect even though it may be narrower in other respects. (See MPEP 1412.03).

B. Claims 44-64 are rejected under 35 U.S.C. 251 as being improperly broadened in a reissue application made and sworn to by the assignee. The application for reissue may be made and sworn to by the assignee of the entire interest only if the application does not seek to enlarge the scope of the claims of the original patent or, for reissue applications filed on or after September 16, 2012, the application for the original patent was filed by the assignee of the entire interest under 37 CFR 1.46. See MPEP 1412.03 and 1414.

The newly added claims 44-64 in this reissue application recite “an injectable solution comprising an antisense oligonucleotide;” and “the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69);” and wherein the injectable solution is “formulated for” different routes of administration (i.e., parenteral, intravenous, intramuscular,

Application/Control Number: 15/645,842

Page 5

Art Unit: 3991

subcutaneous, intraocular, oral or transdermal administration). The scope of the original claim “isolated antisense oligonucleotide” has been broadened by changing the annealing site of the antisense molecule. A claim is broader in scope than the original claims if it contains within its scope any conceivable product or process which would not have infringed the original patent. A claim is broadened if it is broader in any one respect even though it may be narrower in other respects.

Accordingly, in this broadening reissue application the Reissue Declaration by the assignee filed on 12/12/17 is improper.

C. The reissue oath/declaration filed with this application is defective because it fails to identify at least one error which is relied upon to support the reissue application. See 37 CFR 1.175 and MPEP § 1414.

According to Rule 37 CFR 1.175, the reissue declaration fails to specifically identify at least one error pursuant to 35 USC 251 being relied upon as basis for the reissue.

A statement in the oath/declaration of “...failure to include a claim directed to ...” and then reciting all the limitations of a newly added claim, would not be considered a sufficient “error” statement because applicant has not pointed out what the other claims lacked that the newly added claim has, or vice versa. Such a statement would be no better than saying in the reissue oath or declaration that “this application is being filed to correct errors in the patent which may be noted from the change made by adding new claim 10.” In both cases, the error has not been identified. See MPEP 1414(II)(c ).

Further, according to 37 CFR 1.175 (b) the reissue declaration fails to identify a claim that the applicant seeks to broaden.

2. Claims 44-64 are rejected as being based upon a defective reissue Oath and Declaration under 35 U.S.C. 251 as set forth above. See 37 CFR 1.175.

The nature of the defect(s) in the Declaration is set forth in the discussion above in this Office action.

### *Claims and Claim Interpretation*

Application/Control Number: 15/645,842

Page 6

Art Unit: 3991

Original claims 1-43 of the US Patent 8,455,636 are canceled. New claims 44-64 are currently pending in this reissue application and all claims recite "an injectable solution comprising an antisense oligonucleotide" and the independent claims differ from each other by reciting different routes of administration of the antisense molecule. Claim 44 is reiterated below:

44. (New) An injectable solution comprising:

an antisense oligonucleotide of 25 bases comprising a base sequence that is 100% complementary to 25 consecutive nucleotides of a target region of exon 53 of the human dystrophin pre-mRNA, wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69), wherein the antisense oligonucleotide base sequence comprises at least 20 consecutive bases of C AUU CAA CUG UUG CCU CCG GUU CUG AAG GUG (SEQ ID NO: 193), in which the uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, wherein the antisense oligonucleotide is chemically linked to a polyethylene glycol chain, and wherein the antisense oligonucleotide specifically hybridizes to the target region to induce exon 53 skipping; and

a pharmaceutically acceptable carrier or diluent; wherein the injectable solution is formulated for intravenous administration.

#### **MPEP 2111:**

During patent examination, the pending claims must be "given their broadest reasonable interpretation consistent with the specification." The Federal Circuit's *en banc* decision in *Phillips v. AWH Corp.*, 415 F.3d 1303, 1316, 75 USPQ2d 1321, 1329 (Fed. Cir. 2005) expressly recognized that the USPTO employs the "broadest reasonable interpretation" standard:

The Patent and Trademark Office ("PTO") determines the scope of claims in patent applications not solely on the basis of the claim language, but upon giving claims their broadest reasonable construction "in light of the specification as it would be interpreted by one of ordinary skill in the art." *In re Am. Acad. of Sci. Tech. Ctr.*, 367 F.3d 1359, 1364[, 70 USPQ2d 1827, 1830] (Fed. Cir. 2004). Indeed, the rules of the PTO require that application claims must "conform to the invention as set forth in the remainder of the specification and the terms and phrases used in the claims must find clear support or antecedent basis in the description so that the meaning of the terms in the claims may be ascertainable by reference to the description." 37 CFR 1.75(d)(1).

Claims 44-64 recite an injectable solution comprising an antisense oligonucleotide of 25 base sequence; a pharmaceutically acceptable carrier or diluent (or phosphate buffered saline); and the solution is formulated for intravenous administration

Application/Control Number: 15/645,842

Page 7

Art Unit: 3991

According to the new claims the 25 base long antisense molecule includes the following limitations:

1) The antisense oligonucleotide sequence is 100 % complementary to 25 consecutive nucleotides of a target region of exon 53 of the human dystrophin pre-mRNA, and the target region is within annealing site H53A(+23+47) and H53A(+39+69);

2) The antisense oligonucleotide base sequence comprises at least 20 consecutive bases of SEQ ID NO: 193, in which uracil bases are thymine bases;

3) The antisense oligonucleotide is a morpholino antisense oligonucleotide;

4) The antisense oligonucleotide is chemically linked to a polyethylene glycol chain;

5) The antisense oligonucleotide specifically hybridizes to the target to induce exon 53 skipping.

The specification discloses that for exon skipping in exons of Dystrophin gene transcript, the antisense molecules are preferably selected from the group of compounds shown in Table 1A (col. 23, lines 33-36). The specification discloses an antisense oligonucleotide of sequence SEQ ID NO: 193, which is complementary to an exon 53 target annealing site H53A(+39+69); and an antisense oligonucleotide of sequence SEQ ID NO: 195 which is complementary to an exon 53 target annealing site H53A(+23+47) (see Table 1A) (see columns 17-18). The specification discloses that the antisense molecule H53A(+39+69) (SEQ ID NO: 193) was able to induce strong exon skipping to 50 nM (Table 39 in column 56); and antisense molecule H53A(+23+47) (SEQ ID NO: 195) showed very faint exon skipping (Table 39, column 56).

The claimed 25 base long antisense molecule is 100 % complementary to 25 consecutive nucleotides of target region of exon 53 of human dystrophin pre-mRNA; and also the antisense molecule comprises at least 20 consecutive bases of SEQ ID NO: 193. The claim further recites that the target region is “within” the two annealing sites H53A(+23+47) and H53A(+39+69). However, the specification nowhere discloses the target region within the two annealing sites as recited in the claims. The target region within the annealing sites H53A(+23+47) and H53A(+39+69) is 9 base long, whereas the claimed antisense molecule is 25 base long and is 100 % complementary to 25 consecutive nucleotides of the target region, and comprises at least

Application/Control Number: 15/645,842

Page 8

Art Unit: 3991

20 consecutive bases of the sequence of SEQ ID NO: 193. The specification does not specifically disclose an antisense molecule that hybridizes to the 25 consecutive nucleotides of a target region within the annealing site H53A(+23+47) and H53A(+39+69).

The specification discloses that the antisense molecule is chemically linked to polyethylene glycol (col. 27, lines 27-39).

The specification discloses that the antisense molecule is admixed with a pharmaceutically acceptable carrier, diluent, or excipient (col. 28, lines 22-24, lines 41-44; col. 29, lines 58-64). The pharmaceutically acceptable carrier refers to a diluent, adjuvant, excipient, or a vehicle (col. 28, lines 29-31). Water or saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in Martin, Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing o., Easton, Pa (1990) (col. 28, lines 34-39).

The specification discloses that the preferred pharmaceutical compositions comprising the antisense molecules are administered by injection, orally, or pulmonary, or nasal route (col. 28, lines 64-66); the antisense molecules are delivered by intravenous, intra-arterial, intraocular, intraperitoneal, intramuscular, or subcutaneous routes of administration (col. 28, line 66 to col. 29, line 2; col. 30, lines 58 to col. 31, lines 2). The specification further discloses that the "routes of administration described are intended only as a guide since a skilled practitioner will be to determine readily the optimum route of administration and any dosage for any particular animal and condition" (see Col. 29, line 65 to col. 30 line 1).

***Statement Under 37 CFR 3.73 ( c )***

This application is objected to under 37 CFR 1.172(a) as the assignee has not established its ownership interest in the patent for which reissue is being requested. An assignee must establish its ownership interest *in order to support the consent to a reissue application required by 37 CFR 1.172(a)*. The assignee's ownership interest is established by:

(a) filing in the reissue application evidence of a chain of title from the original owner to the assignee, or

Application/Control Number: 15/645,842

Page 9

Art Unit: 3991

(b) specifying in the record of the reissue application where such evidence is recorded in the Office (e.g., reel and frame number, etc.).

The submission with respect to (a) and (b) to establish ownership must be signed by a party authorized to act on behalf of the assignee. See MPEP § 1410.01.

The Statement under 37 CFR 3.73 (c ) filed on 7/10/17 is objected for the following reasons:

- 1) The statement does not identify the Patent number;
- 2) The Assignment Reel/frame number listed does not correspond to the reel/frame number recorded of the present patent.

The office assignment record shows Reel/Frame: 044163/0551 which is different from the listed 030124/0291 on the 3.73 form filed on 7/10/17.

An appropriate paper satisfying the requirements of 37 CFR 3.73 must be submitted in reply to this Office action.

### *Interference estoppel*

(1) *Estoppel*. A judgment disposes of all issues that were, or by motion could have properly been, raised and decided. A losing party who could have properly moved for relief on an issue, but did not so move, may not take action in the Office after the judgment that is inconsistent with that party's failure to move, except that a losing party shall not be estopped with respect to any contested subject matter for which that party was awarded a favorable judgment. See 37 CFR 41.127 (a) (1).

If a party loses on an issue, it may not re-litigate the issue before the examiner or in a subsequent Board proceeding. The time for the party to make all pertinent arguments is during the interference, unless the Board expressly prevented the party from litigating the issue during the interference. See MPEP 2308.03.

There are two main types of interference estoppel. First, a losing party is barred on the merits from seeking a claim that would have been anticipated or rendered obvious by the subject matter of the lost count. See *In re Deckler*, 977 F.2d 1449, 24 USPQ2d 1448 (Fed. Cir. 1992); and *Ex parte Tytgat*, 225 USPQ 907 (Bd. Pat. App. & Inter. 1985). Second, a losing party is procedurally barred from seeking from the examiner relief that could have been--but was not--sought in the interference. See 37 CFR 41.127(a)(1); and *Ex parte Kimura*, 55 USPQ2d 1537 (Bd. Pat. App. & Inter. 2000) (reissue applicant estopped to claim compound when patentability of that compound could have been put in issue in interference where opponent's application also described compound). See MPEP 2308.03.

Application/Control Number: 15/645,842

Page 10

Art Unit: 3991

The present reissue Patent 8,455,636 (University of Western Australia) was the junior party in the Interference against Patent Application 11/233,495 (now issued US 9,896,687, Senior party) (see Patent Interference No. 106,007).

PTAB Ordered that judgment be entered against Junior party University of Western Australia; Ordered that claims 1-43 (all claims) of UWA's involved patent 8,455,636 be cancelled (see April 29, 2016 decision).

In the present reissue application of the '636 patent, applicants canceled the original claims 1-43 and added new claims 44-64.

The original claims 1-43 in the '636 patent are drawn to an isolated antisense oligonucleotide; a pharmaceutical composition comprising the antisense oligonucleotide; and a method of treating Duchenne muscular dystrophy. The relevant claims in the '636 patent are reiterated below.

1. An isolated antisense oligonucleotide of 20 to 50 nucleotides in length comprising at least 20 consecutive nucleotides of SEQ ID NO: 193, wherein the oligonucleotide specifically hybridizes to an exon 53 target region of the human dystrophin gene inducing exon 53 skipping, and wherein the uracil bases are optionally thymine bases

8. The antisense oligonucleotide of claim 7, wherein the non-natural moieties are morpholinos.

16. The antisense oligonucleotide of claim 16, wherein the oligonucleotide is chemically linked to a polyethylene glycol chain.

19. An isolated antisense oligonucleotide of 20 to 50 nucleotides in length comprising at least 20 consecutive nucleotides complementary to an exon 53 target region of the human dystrophin gene designated as annealing site H53A(+ 39+69), wherein the antisense oligonucleotide specifically hybridizes to the annealing site inducing exon 53 skipping, and wherein uracil bases in the antisense oligonucleotide are optionally thymine bases.

21. The antisense oligonucleotide of claim 19, wherein the uracil bases are thymine.

24. The antisense oligonucleotide of claim 19, wherein the sugar moieties of the oligonucleotide backbone are replaced with non-natural moieties.

25. The antisense oligonucleotide of claim 24, wherein the non-natural moieties are morpholinos.

33. The antisense oligonucleotide of claim 19, wherein the oligonucleotide is chemically linked to one or more moieties or conjugates that enhance the activity, cellular distribution, or cellular uptake of the antisense oligonucleotide.

35. The antisense oligonucleotide of claim 33, wherein the oligonucleotide is chemically linked to a polyethylene glycol chain

36. A pharmaceutical composition, comprising an anti-sense oligonucleotide of claim 1, and a saline solution that includes a phosphate buffer.

38. The antisense oligonucleotide of claim 1, comprising SEQ ID NO: 193, wherein the uracil bases are thymine bases.

41. A pharmaceutical composition, comprising an anti-sense oligonucleotide of claim 19, and a saline solution that includes a phosphate buffer.

42. A pharmaceutical composition, comprising an anti-sense oligonucleotide of claim 38, and a saline solution that includes a phosphate buffer.

Application/Control Number: 15/645,842

Page 11

Art Unit: 3991

43. A method of treating Duchenne muscular dystrophy, comprising administering to a patient in need thereof an effective amount of a pharmaceutical composition of claim 36.

The newly added claims 44-64 in this reissue application include several independent claims. The representative independent claim 44 is reiterated below.

44. (New) An injectable solution comprising:

an antisense oligonucleotide of 25 bases comprising a base sequence that is 100% complementary to 25 consecutive nucleotides of a target region of exon 53 of the human dystrophin pre-mRNA, wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69), wherein the antisense oligonucleotide base sequence comprises at least 20 consecutive bases of C AUU CAA CUG UUG CCU CCG GUU CUG AAG GUG (SEQ ID NO: 193), in which the uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, wherein the antisense oligonucleotide is chemically linked to a polyethylene glycol chain, and wherein the antisense oligonucleotide specifically hybridizes to the target region to induce exon 53 skipping; and

a pharmaceutically acceptable carrier or diluent; wherein the injectable solution is formulated for intravenous administration.

The newly claimed “injectable solution” comprises an antisense molecule, which is chemically linked to a polyethylene glycol chain; and the injectable solution (composition) includes a pharmaceutically acceptable carrier or diluent, or phosphate-buffered saline. New claims further recite that the “injectable solution is formulated for” different routes of administration, which is considered as intended use. Even though new claims 44-64 recite an injectable solution, the antisense oligonucleotide molecule in the injectable solution is same as the original claims (see the ‘636 patent claims) since the antisense molecule comprises at least 20 consecutive bases of the sequence of SEQ ID NO: 193; original claims also recite a “pharmaceutical composition” comprising the antisense oligonucleotide in a phosphate buffered-saline (a pharmaceutically acceptable carrier) (see the ‘636 patent claims 36, 41 and 42); and the antisense oligonucleotide is chemically linked to a polyethylene glycol (see the ‘636 patent claims 18 and 35); and the pharmaceutical composition is administered to a patient in the ‘636 patent claim 43. The recitation that the injectable solution is “formulated for” for different routes of administration does not make the claims distinct from the original claims, since the original claims recite a “pharmaceutical composition” (claims 41 and 43) and a method for

Application/Control Number: 15/645,842

Page 12

Art Unit: 3991

“administering to a patient” the pharmaceutical composition (claim 43). Further the limitation “formulated for” does not result in a structurally different composition since the specification does not show that the pharmaceutical composition of the original claims is structurally different from the “injectable solution formulated for different routes of administration” and since the recitation “formulated for” different routes of administration in new claims is considered as intended use limitation. Further in this reissue application, applicants asserted that support for new claims can be found in the ‘636 patent claims (see 7/10/17 amendment).

Accordingly, all the limitations of the new claims were present in the original claims.

The limitations “injectable solution” and “formulated for different routes of administration” in the new claims do not result in a structurally different product as compared to the original claims. The specification neither discloses a definition of “injectable solution” nor discloses that the pharmaceutical composition is structurally or chemically different from the injectable solution or recites additional ingredients which are specific to the injectable solution. The ‘636 patent specification discloses that the pharmaceutical compositions of the present invention may be administered by injection, orally, or pulmonary or nasal route (col. 28, lines 64-66). The ‘636 patent specification discloses that the pharmaceutical compositions are administered by the routes of administration known in the art (see col. 28, lines 62 to column 29, lines 67). Thus the ‘636 patent specification does not disclose that the “injectable solution” which is “formulated for” different routes of administration includes structurally/chemically different composition as compared to the pharmaceutical composition claimed in the ‘636 patent. Accordingly, new claims 44-64 are not patentably distinct from the subject matter of the lost count.

Claims 44-64 are rejected based on estoppel under 37 CFR 41.127(a) (1) for failure to take action during interference 106.007 to place the subject matter of claims 44-64 in interference and (2) based on rationale of *In re Deckler*.

The present reissue application introduces two types of interference estoppel.

1) A losing party is barred on the merits from seeking a claim that would have been anticipated or rendered obvious by the subject matter of the lost count.

Application/Control Number: 15/645,842  
Art Unit: 3991

Page 13

2) A losing party is procedurally barred from seeking from the examiner relief that could have been--but was not--sought in the interference. See 37 CFR 41.127(a)(1).

An applicant may file a motion during the interference to add or amend a claim. A patentee may file a reissue application in support of a motion to add or amend a claim. See MPEP 2308.02.

After the Interference decision (4/29/16), Patentee files this reissue application with new claims. New claims 44-64 are not patentably distinct from the subject matter of the lost count.

A patentee may file a reissue application in support of a motion to add or amend a claim (MPEP 2308.02). The time to add new claims or file a reissue with added claims was during the interference in support of a motion filed during the interference. However, Patent Owner of the '636 patent failed to file a motion to add or amend a claim during the interference 106,007.

Additionally for the reasons discussed above, claims 44-64 are not patentably distinct from the subject matter of the lost count. Thus, present reissue claims 44-64 are rejected as estopped on the merits by the applicant's loss in interference.

### ***Claim Rejections***

1. The following is a quotation of the first paragraph of 35 U.S.C. 112(a):  
(a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:  
The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

2. Claims 44-64 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention.

Application/Control Number: 15/645,842  
Art Unit: 3991

Page 14

The specification fails to show that applicant has possession of an antisense molecule which is 25 base long and is 100 % complementary to a 25 bases of a target region within the annealing sites H53A(+23+47) and H53A(+39+69). The antisense molecule comprises at least 20 consecutive bases of sequence of SEQ ID NO: 193. Claims 44-64 recite a 25 base long antisense molecule, which is 100 % complementary to 25 consecutive nucleotides of target region of Exon 53 of human dystrophin pre-mRNA; and also the antisense molecule comprises at least 20 consecutive bases of SEQ ID NO: 193, and claims further recite that the target region is “within” the two annealing sites H53A(+23+47) and H53A(+39+69). The specification teaches neither the target region within the two annealing sites H53A(+23+47) and H53A(+39+69) nor the antisense molecule which binds to the target region within the two annealing sites.

The specification in Table 1A discloses the antisense molecules that are targeted to the target region of human dystrophin exon 53 acceptor sites. The specification discloses the antisense molecule H53A(+39+69) of SEQ ID NO: 193 (Table 1A; Figure 22; Col. 56 and Table 39); the antisense molecule H53A(+23+47) of SEQ ID NO: 195 (Table 1A; Col. 56 and Table 39). The specification fails to show that applicant has possession of an antisense molecule that hybridizes to the 25 consecutive nucleotides of a target region within the annealing site H53A(+23+47) and H53A(+39+69). The specification further teaches at the time of filing the antisense molecules that induce exon skipping are not always successful (see col. 3); and further teaches that the choice of target selection plays a crucial role in the efficiency of exon skipping (col. 4, lines 21-26). Accordingly, a person skilled in the art would consider exon skipping is unpredictable art at the time of filing of the invention. Thus, in view of unpredictability in the art and absence of sufficient disclosure in the specification a person skilled in the art would not reasonably predict the sequence of the antisense molecule which is 100 % complementary to a target region within the cited annealing sites. For these reasons claims 44-64 are not supported by adequate written description in the '636 patent disclosure.

3. The following is a quotation of 35 U.S.C. 112(b):  
(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

Application/Control Number: 15/645,842

Page 15

Art Unit: 3991

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

4. Claims 44-64 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

The new claims 44-64 recite that the target region is “within” the two annealing sites H53A(+23+47) and H53A(+39+69). The target region within the annealing sites H53A(+23+47) and H53A(+39+69) is 9 base long (i.e., 47-39), whereas the claimed antisense molecule is 25 base long and is 100 % complementary to 25 consecutive nucleotides of the target region, and comprises at least 20 consecutive bases of the sequence of SEQ ID NO: 193. Since, the target region within the two listed annealing sites is 9 base long, it is not clear how 25 base oligonucleotide would be 100% complementary to 25 consecutive nucleotides of the target region. It is not clear whether the target region is within the two claimed annealing sites (which is 9 base long) or the antisense molecule anneals to only H53A(+39+69) since the claims also recite that the base sequence of the antisense molecule comprises at least 20 consecutive bases of sequence of SEQ ID NO: 193. Thus, meets and bounds of the claimed antisense molecule is not clear.

5. The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

6. Claims 44-64 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over WO 2004/0834321 (VO), Koenig (Cell, Vol. 53, Issue 2. 1988, pages 219-228), Koenig et al (Cell, Volume 50, Issue 3, 31 July 1987, Pages 509-517) and WO 2001/72765 (Bennett).

Note: this rejection is based on the claim interpretation that the injectable solution of the present claims comprises a 25 base long antisense molecule, which is 100 % complementary to 25 consecutive nucleotides of target region of Exon 53 of human dystrophin pre-mRNA; and also the antisense molecule comprises at least 20 consecutive bases of SEQ ID NO: 193.

Application/Control Number: 15/645,842  
Art Unit: 3991

Page 16

VO discloses an antisense oligonucleotide molecule h53AON1 (see Table 2 in page 48), which hybridizes to the target region to induce exon 53 skipping. h53AON1 is a 18 nucleobase sequence: **cuguugccuccgguucug**. The antisense oligonucleotide (AON) of the present claims include at least 20 bases of the sequence of SEQ ID NO: 193 (annealing site H53A(+39+69)), which is 31 nucleobase long sequence, which includes the 18 bases of h53AON1. The sequences are compared below with the identical portions shown in bold:

SEQ ID 193 (31mer): **cauuc aacug uugcc uccgg uucug aaggx g**  
VO (h53AON1 18mer): **cug uugcc uccgg uucug**

h53AON1 differs from the claimed AON sequence by having only 18 nucleobases instead of the required “at least 20 bases” of the sequence of SEQ ID NO: 193. However, VO teaches antisense oligonucleotides that are preferably complementary to a consecutive part of between 16 to 50 nucleotides of said RNA (page 9). Koenig (1988) and Koenig (1987) teach the complete sequence of the human Duchenne muscular dystrophy (DMD) cDNA. Thus, it would have been obvious to a person of ordinary skill in the art at the time of the invention to make AONs having different lengths that include the sequence of h53AON1 but still complementary to exon 53. It would have been obvious to modify the disclosed h53AON1 to cause exon skipping by changing its sequence length by adding additional nucleobases, since VO teaches making complementary AONs of various lengths and specifically teaches antisense oligonucleotides that are preferably complementary to a consecutive part of between 16 to 50 nucleotides of said RNA (page 9). Thus, a person of ordinary skill in the art would have been motivated to investigate AONs of different lengths in order to determine the optimum length for exon skipping. A person having ordinary skill in the art would have a reasonable expectation of success that exon skipping would be maintained when a small number of complementary nucleobases (two bases) are added to the h53AON1.

Further, the instant claims recite an injectable solution comprising the AON and a pharmaceutically acceptable carrier or phosphate buffered saline. At the time of the invention it was well known in the art to formulate the AONs with a pharmaceutically acceptable carrier, diluent or PBS for different routes of administration. VO specifically teaches pharmaceutical

Application/Control Number: 15/645,842

Page 17

Art Unit: 3991

preparations comprising the AONs for treatment of inherited diseases (page 17). Further, Bennett in the same field of endeavor teaches that the pharmaceutical compositions and formulations which include antisense compounds of the invention are administered by oral and parenteral administration including intravenous, intra-arterial, subcutaneous, peritoneal or intramuscular injection (see page 25). Bennett further teaches that the formulations for the parenteral administration include buffers, diluents and other pharmaceutically acceptable carriers and excipients (page 26). Bennett teaches suitable pharmaceutically acceptable excipient such as polyethylene glycol can be used to formulate the AONs compounds (pages 47, 48). Bennet also teaches pharmaceutical compositions formulated in liposomes (page 26, 35-40). Thus, it would have been obvious to a person of ordinary skill in the art at the time of the invention to prepare injectable pharmaceutical preparations comprising the AONs for administration by known administrative routes. Regarding “the uracil bases are thymine bases,” VO teaches that any oligonucleotide fulfilling the requirements of the invention may be used to induce exon skipping in the DMD gene (see page 9). The “oligonucleotide” in the VO encompasses both DNA and RNA nucleic acid as well as nucleic acids that are the combination of RNA and DNA. Since uracil bases are normally associated with RNA, and thymine bases are normally associated with DNA, VO clearly teaches the use of thymine bases in place of uracil bases. VO teaches that the AONs comprise morpholino phosphorimidate (page 10).

### ***Conclusion***

Claims 44-64 in this reissue application are rejected. Claims 1-43 of the US patent 8,455,636 are canceled.

### ***Future Correspondences***

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Padmashri Ponnaluri whose telephone number is 571-272-0809. The examiner can normally be reached on Monday through Friday between 8 AM and 4.30 PM. If attempts to reach the examiner by telephone are unsuccessful, the examiner’s supervisor Jean Witz can be reached on 571-272-0927.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR

Application/Control Number: 15/645,842

Page 18

Art Unit: 3991

system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

All correspondence relating to this Reissue proceeding should be directed to:

**By EFS:**

Registered users may submit via the electronic filing system EFS-Web at

<https://efs.uspto.gov/efile/myportal/efs-registered>

**By Mail to:**

Attn: Mail Stop "Ex Parte Reexam"  
Central Reexamination Unit  
Commissioner for Patents  
P. O. Box 1450  
Alexandria VA 22313-1450

**By FAX to:**

(571) 273-9900  
Central Reexamination Unit

**Hand-Deliver any communications to:**

Customer Service Window  
Attn: Central Reexamination Unit  
Randolph Building, Lobby Level  
401 Dulany Street  
Alexandria, VA 22314

/Padmashri Ponnaluri/  
Patent Reexamination Specialist  
Central Reexam Unit 3991

/Johnny F. Railey II/  
Patent Reexamination Specialist  
Central Reexam Unit 3991

/Jean C. Witz/  
Supervisory Patent Reexamination Specialist  
Central Reexam Unit 3991

Reply to Office Action of July 12, 2018

WILTON *et al.*  
Application No. 15/645,842

***Remarks***

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendment, claims 44-64 are pending in the application, with claims 44, 46, 47, 49, 50, 52, 53, 55, 56, 58, 59, 61, 62 and 64 being the independent claims. Claims 44, 46, 47, 49, 50, 52, 53, 55, 56, 58, 59, 61, 62 and 64, as originally presented in the reissue application are sought to be amended to more clearly claim the invention.

Each of the amended claims is amended to recite "a morpholino antisense oligonucleotide 25 bases in length" in place of "antisense nucleotide of 25 bases." Each of the amended claims is also amended to recite "said morpholino antisense oligonucleotide chemically linked to a polyethylene glycol chain" after the term "human dystrophin pre-mRNA." In view of these amendments, the clauses "wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide," and "wherein the antisense oligonucleotide is chemically linked to a polyethylene glycol chain," are deleted. Additionally, each of the amended claims is amended to delete, without prejudice, the phrase "wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)." And each of the amended claims is amended to recite "wherein the antisense oligonucleotide specifically hybridizes to the target region and induces exon 53 skipping" in place of "wherein the antisense oligonucleotide specifically hybridizes to the target region to induce exon 53 skipping." Support for the amendments to the claims can be found throughout the specification, for example, at col. 56, line 19 – col. 58, line 16; Table 1A, col. 17; col. 25, lines 43-52; col. 24, lines 45-48; and col. 28, lines 19-24.

These changes are believed to introduce no new matter, and their entry is respectfully requested.

Reply to Office Action of July 12, 2018

WILTON *et al.*  
Application No. 15/645,842

Based on the above amendment and the following remarks, Applicant respectfully requests that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

***Statement of the Substance of the Interview***

Applicant thanks Examiners Ponnaluri, Railey and Witz for the courtesy of an in-person interview on September 27, 2018. During the interview, Applicant provided an overview of Sarepta Therapeutics, Inc. ("Sarepta"), the exclusive licensee of the current application, Sarepta's product eteplirsén and its drug candidate golodirsén and Duchenne muscular dystrophy ("DMD"), the disease that eteplirsén and golodirsén are intended to treat. Applicant and the Examiners discussed the outstanding rejections, the Patent Trial and Appeal Board's ("Board's") decision in Interference No. 106,007<sup>1</sup> ("the '007 Interference") regarding U.S. Patent Number 8,455,636 ("the '636 patent"), the van Ommen reference (WO2004/083432; "VO") and the Wood declaration submitted during the interference. The Examiners agreed to consider Applicant's arguments which are included in the response below.

The pending claims in the captioned reissue application are directed to narrow embodiments of an antisense oligonucleotide that specifically hybridizes to a target region of dystrophin exon 53 and induces exon 53 skipping. The pending reissue claims include a number of limitations that provide for patent eligible subject matter and clearly define over the interference count and the art cited during the interference and cited in the Office Action.

---

<sup>1</sup> University of Western Australia ("UWA"), Junior Party v. Academisch Ziekenhuis Leiden ("AZL"), Senior Party.

Reply to Office Action of July 12, 2018

WILTON *et al.*  
Application No. 15/645,842

The Applicant had judgment entered against it in the '007 Interference. The count in the interference was a combination of the two parties' broadest claims:

- UWA Claim 1: An isolated antisense oligonucleotide of 20 to 50 nucleotides in length comprising at least 20 consecutive nucleotides of SEQ ID NO:193, wherein the oligonucleotide specifically hybridizes to an exon 53 target region of the human dystrophin gene inducing exon 53 skipping, and wherein the uracil bases are optionally thymine bases.
- AZL Claim 15<sup>2</sup>: An isolated antisense oligonucleotide of 15 to 80 nucleotides in length comprising at least 15 bases of the sequence cuguugccuccgguucug (SEQ ID NO:29, wherein said oligonucleotide inducing exon 53 skipping in the human dystrophin pre-mRNA, said oligonucleotide comprising a modification selected from the group consisting of 2'-O-methyl, 2'-O-methyl, phosphorothioate, a morpholine ring, a phosphorodiamidate, a peptide nucleic acid and a locked nucleic acid.

See Paper 1, page 4, Int. No. 106,007.

At the conclusion of the interference, all but claim 77 of AZL's application were held to be unpatentable under 35 U.S.C. 112, first paragraph. All of UWA's (the present Applicant's) patent claims were held to be unpatentable, either over prior art or as not being directed to patent eligible subject matter. As AZL had a single claim judged patentable by the Board, and the Applicant had none, judgement was entered against Applicant. See Paper 476, Decision on Motions ("Interference Decision"), page 51, line 14 – page 52, line 8, Int. No. 106,007.

---

<sup>2</sup> The AZL application at issue in the '007 Interference, U.S. Application No. 11/233,495, claims priority to the VO reference applied in the current obviousness rejection.

Reply to Office Action of July 12, 2018

WILTON *et al.*  
Application No. 15/645,842

The Board's holdings were premised on the particular findings and conclusions:

In judging the sufficiency of disclosure for AZL's claims, the Board found that exon skipping is unpredictable based on length, so a person of ordinary skill in the art ("POSA") would have had no reasonable expectation of success in maintaining exon skipping when adding more than a few nucleobases to the AON. See Interference Decision, page 44, lines 4-12.

"We recognize, as we detailed above, that there is a significant degree of unpredictability in the effect of AON sequence length on the ability and efficiency of an AON to cause exon skipping."  
*Id.*, page 41, lines 19-22.

"However, those working in the art were also aware that a degree of exon skipping capability would likely be maintained due to a change in a small number of complementary nucleobases of an AON known to cause skipping."

*Id.*, at lines 22-25.

"[O]ne having ordinary skill in the art would have had a reasonable expectation of success that exon skipping would be maintained when a small number of complementary nucleobases are added to h53AON1."

*Id.*, page 42, lines 1-3.

The significance of this finding is that AZL's claim 77, narrowly directed to the antisense oligonucleotide sequence h53AON1, was the only involved claim in the interference to not be found unpatentable. It is this same h53AON1 from VO that is cited in the current obviousness rejection. Applicant's '636 patent, involved in the interference, only included generic claims.

During the interference, it was not possible to add claims to the issued '636 patent. Moreover, as described below, interference case law does not allow a patentee to file a reissue, and

Reply to Office Action of July 12, 2018

WILTON *et al.*  
Application No. 15/645,842

then file one or more motions during the interference to establish that claims of the reissue application do not correspond to the count, i.e., are directed to a patentably distinct invention.

The present reissue application presents narrow claims reciting a specific combination of elements. The claimed antisense oligonucleotides are:

25 nucleotides/bases in length, seven more than the van Ommen's h53AON1;  
100% complementary to 25 consecutive nucleotides of a target region of exon 53;  
morpholino antisense oligonucleotides chemically linked to a polyethylene glycol chain;  
oligonucleotide specifically hybridizes to the target region to induce exon 53 skipping; and  
provided in a pharmaceutical formulation with specific ingredients.

Additionally, the claimed oligonucleotides are non-obvious over van 'Ommen's 18 base long antisense oligonucleotide having a backbone with 2-O-methyl modified sugar residues; and non-obvious over the interference count.

***Rejections under 36 U.S.C. § 103***

Claims 44-64 are rejected under 35 U.S.C. § 103(a) as unpatentable over van Ommen et al., (WO 2004/083432; VO), Koenig (Cell, 53(2):219-228), 1988), Koenig et al. (Cell, 50(3):509-517, 31 July 1987) and WO 2001/72765 to Bennet et al. Office Action at 15-17. Applicant respectfully disagrees. A POSA would not have had a reasonable expectation from the teachings of the cited references that the claimed 25-mer antisense oligonucleotide would cause skipping of exon 53, as use of antisense oligonucleotides for exon skipping in muscular dystrophy was unpredictable.

The '007 Interference involved the '636 patent and a pending application assigned to AZL with claims directed to antisense oligonucleotides that induce exon 53 skipping for the treatment of

Reply to Office Action of July 12, 2018

WILTON *et al.*  
Application No. 15/645,842

DMD. The independent claims of the '636 patent were directed to an antisense oligonucleotide of 20-50 nucleotides in length. Interference Decision, page 39. Certain claims of the '636 patent were directed to an antisense oligonucleotide containing at least 31 nucleotides. *Id.* at pages 42-43.

During the '007 Interference, Applicant submitted a declaration by Dr. Matthew J.A. Wood, a copy of whose declaration (filed as UWA Exhibit 2081 in Interference No. 106,007; "Wood Declaration") is submitted with this response. The Board relied heavily upon the Wood Declaration in the Interference Decision. In particular, the Board carefully analyzed and summarized the testimony of Dr. Wood, including the discussion of multiple scientific publications showing that it was unpredictable whether an antisense oligonucleotide of a specific length would cause exon skipping in the dystrophin gene. *See* Interference Decision at pages 11-17 discussing the publications presented in ¶¶68-81 of the Wood Declaration.

Dr. Wood concluded that based on these publications, "AONs [antisense oligonucleotides] have an optimal length, which is a result of a number of factors including nucleotide sequence, chemical modifications, and target accessibility, and when that length is either not reached or is exceeded the skipping efficiency drops off." Wood Declaration at ¶74. To support his conclusion, Dr. Wood cited Wu, B., *et al.*, "Targeted skipping of human dystrophin exons in transgenic mouse model systemically for antisense drug development," *PloS one*, 65:e19906 (2011) ("Wu") (Exhibit 2015 to Wood Declaration). According to Dr. Wood, Wu reports that a 20-mer AON induced no detectable exon skipping, but a 22-mer AON induced detectable exon skipping in 4% of cells. *Id.* While adding five nucleotides increased exon skipping to 21%, adding five more nucleotides "largely abrogated this effect." *Id.* The table provided by Dr. Wood illustrating the unpredictability of exon skipping based on oligonucleotide length is reproduced below.

Reply to Office Action of July 12, 2018

WILTON *et al.*  
Application No. 15/645,842

Name	Target	2'-O-Me-PS AON Sequence	Length	Effect
AO3PS	-19+1	UCUUUAAACAGAAAAGCAUAC	20	-
AO4PS	-19+3	CCUCUUUAAACAGAAAAGCAUAC	22	4%
AO5PS	-19+8	AACUCCUCUUUAAACAGAAAAGCAUAC	27	21%
AO6PS	-19+13	CUUCUAAACUCCUCUUUAAACAGAAAAGCAUAC	32	3%

*Id.* Therefore, as Dr. Wood indicated, Wu establishes that changing the length of an antisense oligonucleotide by only five nucleotides can yield very different exon skipping effects.

Based upon the state of the art indicated by Dr. Wood's testimony and the underlying scientific literature, the Board found UWA's dependent claims directed to a particular 31-mer to be nonobvious over VO.<sup>3</sup> Interference Decision at page 44. Specifically, the Board held that "[b]ecause of the unpredictability, we are not convinced that one ordinarily skilled in the art would have had a reasonable expectation of success that an AON having the [claimed sequence] would cause exon skipping." *Id.* In fact, the Board specifically indicated that "[w]e recognize . . . that there is a **significant degree** of unpredictability in the effect of AON sequence length on the ability and efficiency of an AON to cause exon skipping." *Id.* at page 41 (emphasis added). The Board further determined that "a degree of exon skipping capability would likely be maintained due to a change in a small number of complementary nucleobases of an AON known to cause skipping." *Id.* The Board concluded that "[i]t would have been obvious . . . to add the two complementary nucleobases dictated by the known sequence of [the exon at issue] to either end of [the AON at issue] with a reasonable expectation that the resultant 20 base AON would cause exon skipping." *Id.* at page 42. Therefore, the Board concluded that the POSA would have only a reasonable expectation of success

---

<sup>3</sup> The Board found these dependent claims were not directed to patent eligible subject matter under 35 U.S.C. § 101. Interference Decision at page 51.

Reply to Office Action of July 12, 2018

WILTON *et al.*  
Application No. 15/645,842

in maintaining exon skipping if only a small number, *e.g.*, two, nucleobases are added to a particular antisense oligonucleotide.

The "law of the case" doctrine binds the Examiner to apply the Board's determination in this related application. Under the "law of the case" doctrine, the Examiner is bound by the Board's findings. In *Ex Parte Fisher*, No. 2013-000665, at 4, 2015 WL 5782991 (PTAB Sept. 16, 2015), the Board expressly noted that "findings and conclusions in a decision of the Board are the 'law of the case,' and are entitled to issue-preclusive effect during further prosecution." In *Ex Parte Keggenhoff*, No. 2012-001720, at 5, 2014 WL 3950403 (PTAB Aug. 11, 2014), the Board held that an earlier Board appeal decision for the application at issue is "the law of the case." And in *Ex Parte Fong Pong*, No. 2016-002217, at 3, 2017 WL 5714381 (PTAB Nov. 6, 2017), the Board stated, "[c]onsistent with other administrative bodies, the Board has applied the law of the case doctrine and held that all findings and conclusions in a first decision by the Board—whether express or implicit—are entitled to issue-preclusive effect...." Indeed, the Office's Standard Operating Procedure 2 states that "[a PTAB] opinion is binding law of the case, even if it is not designated as precedential, informative, or representative." *See* PATENT TRIAL AND APPEAL BOARD, STANDARD OPERATING PROCEDURE 2 (Revision 9), at 4 (2014). The guidance in the M.P.E.P. further underscores the "law of the case" doctrine, explaining that "a Board decision in an application is the 'law of the case,' and is thus controlling in that application and ***any subsequent, related application.***" M.P.E.P. § 706.07(h)(XI)(A) (emphasis added).

During the '007 Interference, the Board analyzed whether the claims of the '636 patent would have been obvious over VO, applying the same 18-mer (h53AON1) applied in the Office Action in this application. However, the independent claims in the '636 patent recited an antisense

Reply to Office Action of July 12, 2018

WILTON *et al.*  
Application No. 15/645,842

oligonucleotide having a length of 20 to 50 nucleotides, and thus encompassed oligonucleotides 20 bases in length. As discussed above, it was this addition of two bases to the h53AON1 18-mer that the Board found to be obvious. Interference Decision at 42. The Examiner appears to appreciate that the Board's decision was due to the two base difference between h53AON1 in VO and the claims of the '636 patent, as the Office Action states that a "person of ordinary skill in the art would have a reasonable expectation of success that exon skipping would be maintained when a small number of complementary nucleobases (*two bases*) are added to the h53AON1." Office Action at 16, emphasis added.

However, in the current application, the claimed antisense oligonucleotide is 25 bases in length, rather than a range that includes a lower cut-off of 20 bases as it was in the '007 Interference. While the current claims require that the antisense oligonucleotide comprises at least 20 consecutive bases of SEQ ID NO:193, the total length of the claimed antisense oligonucleotide is 25 bases. Seven bases need to be added to h53AON1 to arrive at a 25-mer. There is no indication in the Interference Decision that the Board considers seven bases to be a small number of bases. Instead, as discussed above, the results discussed in the Interference Decision appear to suggest the opposite. *See* Interference Decision at 11-17 discussing the publications presented in ¶¶68-81 of the Wood declaration.

Because a POSA at the time of the invention would have found it unpredictable whether adding seven bases to h53AON1 would have provided an antisense oligomer capable of causing skipping exon 53, the POSA would not have had a reasonable expectation of success in making this change to h53AON1. For at least this reason, the claimed 25-mer would not have been obvious to the POSA over VO in combination with the other cited references.

Reply to Office Action of July 12, 2018

WILTON *et al.*  
Application No. 15/645,842

The obviousness rejection should be withdrawn.

*Not Every Limitation of the Claimed Invention is Found in the Cited Art*

In addition to the above, the cited art fails to disclose each and every limitation of the claimed invention. Specifically, the pending claims all require an antisense oligonucleotide chemically linked to a polyethylene glycol chain. Bennett was cited for teaching pharmaceutical compositions including excipients, such as polyethylene glycol. Office Action at page 17. However, including polyethylene glycol in a pharmaceutical composition *is not* chemically linking an antisense oligonucleotide to a polyethylene glycol chain. Moreover, Bennett does not provide a reason to chemically link a polyethylene glycol chain to an antisense oligonucleotide 25 bases in length comprising a base sequence that is 100% complementary to 25 consecutive nucleotides of a target region of exon 53 of the human dystrophin pre-mRNA, wherein the antisense oligonucleotide base sequence comprises at least 20 consecutive bases of C AUU CAA CUG UUG CCU CCG GUU CUG AAG GUG (SEQ ID NO: 193), in which the uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide. Therefore, for at least this additional reason, the obviousness rejection should be withdrawn.

*Interference estoppel*

Claims 44-64 are rejected based on interference estoppel (a) for failure to take action during Interference No. 106,007 to place the subject matter of the claims in the interference and (b) based on the rationale of *In re Deckler*. Office Action at pages 9-13. Applicant respectfully disagrees.

Reply to Office Action of July 12, 2018

WILTON *et al.*  
Application No. 15/645,842

After losing an interference, a patent applicant is estopped from obtaining claims under two scenarios. The applicant is estopped from obtaining claims that are "not patentably distinct" from the subject matter of an interference count which the Applicant lost (lost count estoppel). And, an applicant is estopped from pursuing claims that were, or could have been raised, in the interference. *In re Deckler*, 977 F.2d 1449, 1451 & 1453-54 (Fed. Cir. 1992). However, for the reasons discussed below, neither "procedural" estoppel nor "lost count" estoppel is applicable here.

### *No Procedural Estoppel*

The Office Action states that the Applicant could have filed a reissue application in support of a motion to add or amend a claim, but failed to do so during the '007 Interference. Office Action at page 13. According to the Office Action, because the Applicant did not file a reissue application with the currently pending claims, the Applicant is procedurally barred from seeking relief in this reissue application. *See id.* However, "the Board does not permit reissue applicants to add claims that would not correspond to a count." M.P.E.P. § 2303C (citing *Winter v. Fujita*, 53 U.S.P.Q.2d 1234, 1249 (Bd. Pat. App. & Inter. 1999)). As discussed below, the currently pending claims are patentably distinct from the lost count and therefore would not have corresponded to the count. Because Applicant could not have added the currently pending claims to the '007 Interference, Applicant did not fail to take action during the Interference and is thus not estopped from pursuing them in the present application.

Reply to Office Action of July 12, 2018

WILTON *et al.*  
Application No. 15/645,842

*Reissue Claims are Patentably Distinct from the Lost Count*

The Office Action states that the current claims are not patentably distinct from the count in the '007 Interference. Office Action at 9-12. The claims are patentably distinct from the count for at least the reasons below.

First, the claims are patentably distinct in that they recite a combination of limitations that is not recited in the count resulting in a claim to a much narrower embodiment of antisense oligonucleotides than that of the count. The claims recite an injectable solution comprising a morpholino antisense oligonucleotide 25 bases in length that is chemically linked to a polyethylene glycol chain. The claimed 25 base antisense oligomer comprises at least 20 consecutive bases of SEQ ID NO: 193 in which the uracil bases are thymine bases. And the claimed antisense oligonucleotide induces exon 53 skipping.

The POSA would not have found the specific combination of limitations recited in the current claims to have been obvious over the count. As pointed out in the reissue declaration:

[I]ssued claims 1-43 of US Patent No. 8,455,636 did not cover an embodiment of an injectable solution comprising an antisense oligonucleotide of 25 bases comprising a base sequence that is 100% complementary to 25 consecutive nucleotides of a target region of exon 53 of the human dystrophin pre-mRNA wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69), wherein the antisense oligonucleotide base sequence comprises at least 20 consecutive bases of C AUU CAA CUG UUG CCU CCG GUU CUG AAG GUG (SEQ ID NO: 193), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, wherein the antisense oligonucleotide is chemically linked to a polyethylene glycol chain, and wherein the antisense oligonucleotide specifically hybridizes to the target region to induce exon 53 skipping; and phosphate-buffered saline, wherein the injectable solution is formulated for intravenous administration.

Reissue Declaration filed December 12, 2017, Appendix A. The Office Action has provided no teaching that would have led the POSA to modify the interference count to combine all of the

Reply to Office Action of July 12, 2018

WILTON *et al.*  
Application No. 15/645,842

elements recited in the currently pending independent claims. Without such a teaching, the claims cannot be obvious over the interference count.

Second, even when looking at individual elements within the claimed combination, there are non-obvious distinctions between the claims and the count. The claimed antisense oligonucleotide is a morpholino antisense oligonucleotide 25 bases in length chemically linked to a polyethylene glycol chain, while the oligonucleotides recited in the count were of any backbone chemistry, having lengths of 20 to 50 nucleotides (from claim 1 of the '636 patent) or 15 to 80 nucleotides (from claim 15 of USSN 11/233,495). The claimed 25-mer represents a small genus that is patentably distinct over the much larger genera represented by the ranges of 20 to 50 nucleotides and 15 to 80 nucleotides. As discussed above in the context of the obviousness rejection, the Board has recognized that, at the time of the invention, it was unpredictable whether an antisense oligonucleotide of a specific length would cause exon skipping in the dystrophin gene. Thus, the POSA would not have had a reasonable expectation that a 25-mer chosen from the ranges recited in the count – 20 to 50 or 15 to 80 – would successfully cause skipping of exon 53.

The claims recite an injectable formulation, which is not recited in the count. The claims also recite a morpholino oligomer that is chemically linked to a polyethylene glycol chain, which is not recited in the count. Further, the claims recite that the antisense oligonucleotide induces exon 53 skipping, which is not recited in the count.

Thus, the current claims have various non-obvious differences from the count in the '007 interference. The POSA would not have found the specific combination of limitations claimed to have been obvious over the count.

Reply to Office Action of July 12, 2018

WILTON *et al.*  
Application No. 15/645,842

For at least the above reasons, the "lost count estoppel" of *In re Deckler* is not applicable and Applicant requests that this rejection be withdrawn.

### ***Claim Amendment***

The Office Action objects to the amendment filed January 29, 2018 as not complying with 37 C.F.R. § 1.173(b) and (c). Office Action at 3. Specifically, the Office Action objects to the previously filed amendments as not providing an explanation of the support for the claim term "target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)." As discussed below, Applicant amends the claims, without prejudice, to delete this phrase. Thus, the objection to the prior amendment is moot.

### ***Rejection of the Reissue Declaration***

The Office Action rejected the reissue declaration filed on December 12, 2017 under 37 C.F.R. § 1.175. Office Action at 3-5.

### ***The Current Amendments Are Not Broadening***

First, the Office Action rejects the reissue declaration on the basis that claims 44-64 are allegedly broadening. Office Action at 4-5, subsections A. and B. Specifically, the Office Action alleges that the terms "an injectable solution comprising an antisense oligonucleotide" and "target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)" are broadening over the previous claims. Applicant respectfully disagrees. These claim terms are not broadening.

Independent claims 1 and 19 of the '636 patent recited "an antisense oligonucleotide" of 20 to 50 nucleotides in length. The currently pending claims recite an injectable solution comprising an

Reply to Office Action of July 12, 2018

WILTON *et al.*  
Application No. 15/645,842

antisense oligonucleotide having a length consisting of 25 bases. Thus, the addition of the term "an injectable solution comprising an antisense oligonucleotide" is not broadening as it limits the claims to antisense oligonucleotides having a length of 25 nucleotides *in an injectable solution*, while the previous claims encompassed an antisense oligonucleotide of 20 to 50 nucleotides in length and were not limited to any type of medium.

Regarding the claim phrase the "target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)," Applicant does not agree that this phrase is broadening. The phrase delineates a target region on exon 53 that falls within two overlapping annealing sites and thus provides a target region spanning from, and including, endpoint H53A+23 to, and including, endpoint H53A+69. Thus, the claim phrase results in a smaller genus than the previous claims. However, solely to advance prosecution of the application, Applicant amends the claims to delete this phrase.

For at least the above reasons, claims 44-64 are not broadening and the reissue declaration is thus proper.

*The Reissue Declaration Properly Identifies an Error*

Second, the Office Action rejects the reissue declaration as allegedly not specifically designating at least one error relied on as the basis for reissue. Office Action at 5. Applicant respectfully disagrees. The reissue declaration filed December 12, 2017 properly identifies at least one error.

The passage in M.P.E.P. § 1414(II)(c) cited in the Office Action states that a reissue declaration fails to identify a sufficient error when "applicant has not pointed out what the other

Reply to Office Action of July 12, 2018

WILTON *et al.*  
Application No. 15/645,842

claims lacked that the newly added claim has, or vice versa." Office Action at 5. But that is not the case here. The reissue declaration specifically points out that:

[I]ssued claims 1-43 of US Patent No. 8,455,636 ***did not cover an embodiment*** of an injectable solution comprising an antisense oligonucleotide of 25 bases comprising a base sequence that is 100% complementary to 25 consecutive nucleotides of a target region of exon 53 of the human dystrophin pre-mRNA wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69), wherein the antisense oligonucleotide base sequence comprises at least 20 consecutive bases of C AUU CAA CUG UUG CCU CCG GUU CUG AAG GUG (SEQ ID NO: 193), in which uracil bases are thymine bases, wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, wherein the antisense oligonucleotide is chemically linked to a polyethylene glycol chain, and wherein the antisense oligonucleotide specifically hybridizes to the target region to induce exon 53 skipping; and phosphate-buffered saline, wherein the injectable solution is formulated for intravenous administration.

Reissue Declaration filed December 12, 2017, Appendix A, emphasis added.

The reissue declaration thus specifically pointed out what the prior claims lacked and thus point out at least one error on which reissue can be based. While the original claims were canceled, the newly added claims are narrower in multiple respects compared to any of the original claims in the '636 patent. The Federal Circuit has found that a reissue that adds only narrower claims without amending any of the original claims is proper. *See In re Tanaka*, 640 F.3d 1246, 1251 (Fed. Cir. 2011).

The Office Action also rejects the reissue declaration as failing to identify a claim that Applicant seeks to broaden. Office Action at 5. But, as discussed above, none of the claims added by Applicant are broadening over the claims of the '636 patent. Thus, this rejection should be withdrawn.

Reply to Office Action of July 12, 2018

WILTON *et al.*  
Application No. 15/645,842

For at least the above, reasons, the reissue declaration filed December 12, 2017 is proper and the rejection of the declaration should be withdrawn. For convenience, Applicant resubmits a copy of the reissue declaration with this reply.

***Rejection under 35 U.S.C. § 251***

The Office Action rejects claims 44-64 under 35 U.S.C. § 251 as allegedly being based upon a defective reissue declaration for the reasons set forth in the section above. Office Action at 5. As claims 44-64 are not broadening and the reissue declaration filed December 12, 2017 is proper, this rejection should be withdrawn.

***Statement Under 37 CFR 3.73(c)***

The Office Action objects to the application under 37 C.F.R. § 1.172(a) as the Office Action alleges that the assignee has not established its ownership interest in the patent for which reissue is being requested. Office Action at 8-9. The objection is based on the fact that Applicant's statement under 37 C.F.R. 3.73 referred to an assignment recorded at Reel/Frame: 030124/0291 while the same assignment is recorded for the current reissue application at Reel/Frame:044163/0551.

Applicant provides copies of two Notices of Recordation in which one executed assignment from the inventors to the University of Western Australia was (1) recorded against the application that matured into the '636 patent (recorded at Reel/Frame: 030124/0291); and (2) recorded against the captioned reissue application (Reel/Frame:044163/0551). The record demonstrated that the assignee, University of Western Australia, has full ownership in the '636 patent, the patent for

Reply to Office Action of July 12, 2018

WILTON *et al.*  
Application No. 15/645,842

which reissue is being requested. A new Statement under 3.373(c) is also filed along with this response to address this objection.

***Rejections under 35 U.S.C. § 112***

***The Claims Have Written Description***

Claims 44-64 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. Office Action at 13-14. Specifically, the Office Action alleges that the claim phrase the "target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)" lacks support in the specification.

Applicant respectfully disagrees with the written description rejection noting that support for the claims can at least be found in Table 39 of the specification. However, solely to advance prosecution of the application, Applicant amends the claims to delete this phrase as it was not present in the claims of the '636 patent. Thus, the written description rejection is moot.

***The Claims Are Definite***

Claims 44-64 are rejected under 35 U.S.C. § 112, second paragraph, as indefinite. Office Action at 14-15. Specifically, the Office Action alleges that the claim phrase the "target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)" is unclear.

Applicant respectfully disagrees with the indefiniteness rejection. The claim phrase the "target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)" is not indefinite. As discussed above, the phrase delineates a target region on exon 53 that falls within two overlapping annealing sites and thus provides a target region spanning from H53A+23 to H53A+69. As the target region has clearly defined boundaries, it is not indefinite. However, solely to advance

- 30 -

Reply to Office Action of July 12, 2018

WILTON *et al.*  
Application No. 15/645,842

prosecution of the application, Applicant amends the claims to delete this phrase. Thus, the indefiniteness rejection is moot.

### ***Conclusion***

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicant therefore respectfully requests that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicant believes that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

/John M. Covert, #38,759/

John M. Covert, Registration No. 38,759  
Eric K. Steffe, Registration No. 36,688  
Attorneys for Applicant

Date: Nov. 2, 2018

1100 New York Avenue, N.W.  
Washington, D.C. 20005-3934  
(202) 371-2600  
10134570\_1.docx

# Exhibit 46

# Grammar Monster (#GM)

Free Grammar Lessons and Exercises



## Non-restrictive Clause

search our site by Craig Shives

This Page Includes...

Printable & Scribble Tool ✓

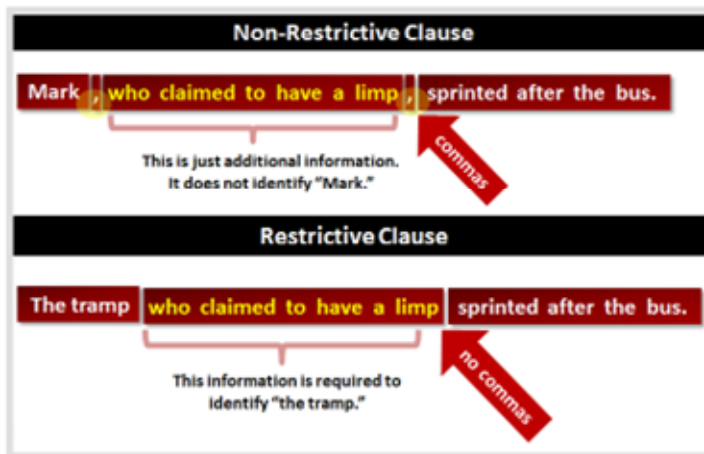
Why Non-restrictive Clauses

Are Important ✓

Easy Practice ✓

### What Is a Non-restrictive Clause? (with Examples)

A non-restrictive clause is a [clause](#) that provides additional, non-essential information. In other words, a non-restrictive clause is not needed to identify the word it modifies, i.e., it's just bonus information. As a non-restrictive clause is not essential to the meaning of a sentence, it is offset with [commas](#) (or some other [parenthetical punctuation](#) such as [dashes](#)).



### Non-restrictive Clauses Contrast with Restrictive Clauses

Here is another example of a non-restrictive clause.

- Peter Jones, who plays goalkeeper for our village football team, has worked at his father's greengrocers for twenty years.  
(The shaded text is a non-restrictive clause. It describes "Peter Jones," but it does not **identify** him. It is merely additional information about him. Deleting this clause would not affect the meaning.)

Non-restrictive clauses contrast with [restrictive clauses](#). Look at this example of a restrictive clause:

- The man who plays goalkeeper for our village football team has worked at his father's greengrocers for twenty years.  
(The bold text is a restrictive clause. It describes "the man," and it **identifies** him. It is not just additional information. It is essential for meaning.)

## Your Choice of Punctuation

You are not limited to commas when offsetting a non-restrictive clause. You can use parentheses (brackets) or dashes too. (See Reason 2 below.)

[Read more about your choices of punctuation for offsetting a non-restrictive clause.](#)

## More Examples of Non-restrictive Clauses

Here are some more examples of non-restrictive clauses:

- I went to London with John Baker, **who lives next door.**  
(This is just additional information. It's a non-restrictive clause.)
- Betty, **who is still on the ferry,** will arrive before 4 o'clock.  
(This is just additional information. It's a non-restrictive clause.)

[Read more about using commas with "which," "that," and "who."](#)

## Some More Examples of Non-restrictive Clauses

Here are some more examples of non-restrictive clauses in real-life quotations (non-restrictive clauses shaded):

- Every journalist has a novel in him, **which is an excellent place for it.**  
(Historian Russell Lynes)
- Humans are the only animals that have children on purpose with the exception of guppies, **who like to eat theirs.** (Journalist P J O'Rourke)
- She had a pretty gift for quotation, **which is a serviceable substitute for wit.**  
(Playwright W. Somerset Maugham)
- You can talk about anything if you go about it the right way, **which is never malicious.** (comedian Rodney Carrington)

## Why Non-restrictive Clauses Are Important

When looking at writing errors, there are more issues associated with [restrictive clauses](#) than non-restrictive clauses. As a general observation, non-restrictive clauses do not cause too many snags. Nevertheless, here are two good reasons to give non-restrictive clauses a little more thought.

### (Reason 1) Know when to use a comma before "who" or "which."

Writers often ask whether to put a comma before "who" and "which." The answer to that question is sometimes yes and sometimes no. It depends whether the "who" or "which" heads a restrictive clause or a non-restrictive clause.

Look at this example:

- My brother, who lives in New York, caught coronavirus.  
(This sentence suggests I have just one brother. I've also told you that he lives in New York, but I could have omitted that information. The shaded text is a non-restrictive clause.)
- My brother **who lives in New York** caught coronavirus.  
(In this sentence, the bold text is a restrictive clause. It specifies that I'm talking about my New York-based brother, i.e., not a different brother.)

Remember that if your adjective clause is essential to identify its noun, then there are no commas. If it's just additional information, use commas, dashes, or parentheses (brackets)...or delete it.

#### Test for a Non-restrictive Clause

If you'd happily put it in parentheses (brackets) or delete it, use commas.




The example above uses "who." Here's one with "which." There is a distinction between "who" and "which" because Brits are okay with a restrictive clause headed by "which," but, as a rule, Americans aren't.

- I've enjoyed the benefits of this country, which has been very good to me.  
(Attorney Wendy Long)  
(This is non-restrictive clause. It does not define the country (the word "this" does that job). The shaded clause is just additional information.)
- How can you govern a country **which has 246 varieties of cheese**? (French President Charles De Gaulle)  
(This is a restrictive clause. It defines the country. Those following British English writing conventions are okay with a restrictive clause headed by "which," but most Americans would use "that" instead of "which."

[Read more about using "who," "which," and "that" \(go to Issue 1 on this link\).](#)

## (Reason 2) Know when to use a comma before "who" or "which."

Commas are the most common type of [parenthetical punctuation](#), but, for certain effects, you can use others. Here are some guidelines:

Choice of Parenthetical Punctuation	Pros and Cons
 commas	<ul style="list-style-type: none"> <li>➢ (pro) normal-looking sentence</li> <li>➤ (con) commas are often confused with other commas in the sentence</li> </ul>
 brackets	<ul style="list-style-type: none"> <li>➢ (pro) parenthesis easily seen</li> <li>➤ (con) brackets make official letters look a little unorganized</li> </ul>
 dashes	<ul style="list-style-type: none"> <li>➢ (pro) parenthesis easily seen</li> <li>➤ (con) dashes look a little stark</li> </ul>

[Read more about your choices of punctuation for offsetting a non-restrictive clause.](#)

## Key Points

- Do you have an adjective clause headed by "which" or "who"? Would you happily put it in parentheses? Yes? It's non-restrictive. Offset it with commas or, if you think it helps, dashes or parentheses (round brackets).
- Knowing your options for parenthetical punctuation is a useful writing tool.

## Printable Test

00

1 not attempted

True or false? A non-restrictive clause is a clause that is not needed to identify the word it modifies.

A. True

B. False

2 not attempted

Select the one with a non-restrictive clause in bold:

A. She admires Mark, **whom she finds helpful.**

B. She admires people **who are helpful.**

3 not attempted

Choose the correct version.

A non-restrictive clause is **essential to the meaning of a sentence.** /  
**just additional information.**

4 not attempted

Select the one with a non-restrictive clause in bold:

A. Is this the boy **who stole your purse?**

B. This is Simon, **who stole your purse.**

5 not attempted

Choose the correct version.

A non-restrictive clause **is** / **isn't** offset with commas.

6 not attempted

Select the one with a non-restrictive clause in bold:

A. She loves her dog, **which has floppy ears.**

B. She loves dogs **that have floppy ears.**

7 not attempted

Select the one with a non-restrictive clause in bold:

A. I want to fish off the pier, **where even Lee could catch a bass.**

B. I know a place **where even Lee could catch a bass.**

8 not attempted

True or false? You can also use brackets or dashes to offset non-restrictive clauses.

A. True

B. False

9 not attempted

Select the one with a non-restrictive clause in bold:

A. The reprimand **you received** was severe.

B. He was jailed for four years, **which was quite harsh.**

10 not attempted

Select the one with a non-restrictive clause in bold:

A. John Baker, **who lives next door,** went to London.

B. The man **who lives next door** went to London.

00



# Exhibit 47

# Complete Cloning of the Duchenne Muscular Dystrophy (DMD) cDNA and Preliminary Genomic Organization of the DMD Gene in Normal and Affected Individuals

M. Koenig,\* E. P. Hoffman,\* C. J. Bertelson,\*  
A. P. Monaco,\*† C. Feener,\*‡ and L. M. Kunkel\*†‡

\* Division of Genetics

Mental Retardation Program

Department of Pediatrics

Harvard Medical School

The Children's Hospital

Boston, Massachusetts 02115

†The Program in Neuroscience

Harvard University

Cambridge, Massachusetts 02138

‡The Howard Hughes Medical Institute

Boston, Massachusetts 02115

## Summary

**The 14 kb human Duchenne muscular dystrophy (DMD) cDNA corresponding to a complete representation of the fetal skeletal muscle transcript has been cloned. The DMD transcript is formed by at least 60 exons which have been mapped relative to various reference points within Xp21. The first half of the DMD transcript is formed by a minimum of 33 exons spanning nearly 1000 kb, and the remaining portion has at least 27 exons that may spread over a similar distance. The DNA isolated from 104 DMD boys was tested with the cDNA for detection of deletions and 53 patients exhibit deletion mutations. The majority of deletions are concentrated in a single genomic segment corresponding to only 2 kb of the transcript.**

## Introduction

Duchenne muscular dystrophy (DMD) is a severe human X-linked degenerative disorder of muscle with fatal evolution before the end of the third decade of life. It affects about 1 in 3500 live born males. The high disease frequency correlates with the high rate of new mutations, estimated to represent one-third of all DMD cases (for review, see Moser, 1984). Both cytogenetically detectable abnormalities (Greenstein et al., 1977; Zatz et al., 1981; Verellen-Dumoulin et al., 1984; Francke et al., 1985) and genetic linkage analysis (Davies et al., 1983; Bakker et al., 1985; Goodfellow et al., 1985) independently localized the DMD locus in the band Xp21. The less severe and less frequent Becker muscular dystrophy (BMD) was localized to the same region (Kingston et al., 1983), and is probably allelic with DMD (Kunkel et al., 1986; Monaco et al., 1987). The DNA of a DMD-deletion patient, B.B. (Francke et al., 1985) was used to isolate 7 pERT clones that were absent from this patient's DNA (Kunkel et al., 1985). One of these deletion-specific clones, pERT87 (DXS164), was found to be tightly linked to the DMD gene and detected deletions in 6.5% of DMD patients (Monaco et al., 1985; Kunkel et al., 1986). The pERT87 clone was the starting point for a 220 kb genomic walk using human recombinant phage

libraries (Monaco et al., 1986). A number of deletions exhibited breakpoints within the 220 kb of walked DNA, and the junctions for some deletions were cloned (clones J-Bir, J-MD, J-47 and J-66; Monaco et al., 1987; Monaco and Kunkel, 1987). Independently, Ray et al. (1985) isolated an X-specific junction clone, XJ1.1(DXS206), from the X:21 translocation chromosome of a female DMD patient, which also detects deletions in DMD patients. Using cloned fragments from Xp21, a long-range restriction enzyme map of the Xp21 region involved in the DMD phenotype was determined (van Ommen et al., 1986; Burmeister et al., 1986; Kenwrick et al., 1987).

The genomic sequences of the DXS164 locus found to be conserved among mammalian species eventually led to the identification of an estimated 16 kb DMD transcript and to the isolation of a corresponding 1 kb human cDNA spanning the DXS164 locus (Monaco et al., 1986). The direction of transcription was predicted to be from centromere to telomere from the orientation of the conserved exon and the determined DNA sequence. We report here the complete cloning of the 14 kb human DMD cDNA and the analysis of the structure of the locus encompassed by these sequences relative to the previously described genomic loci. Parts of the complete DMD cDNA were also used to search for additional deletions and RFLPs in the Xp21 region that would aid in the diagnosis of DMD mutations.

## Results

### Cloning of the cDNA

The original human fetal muscle cDNA library used to clone a portion of the DMD gene contained many small inserts, some of which contained portions representative of other RNA transcripts. In an effort to facilitate the cloning of the entire DMD cDNA, a new human fetal skeletal muscle cDNA library was constructed in the phage vector lambda gt10. Approximately  $1.1 \times 10^6$  primary unamplified phage clones were hybridized with two cDNA probes representing the most extreme DMD cDNA fragments available at the time of the screen. Ten clones were obtained with the 5' probe and 23 with the 3' probe, of which six and eight clones, respectively, were further analyzed. Restriction enzyme cleavage of the cDNA inserts indicated that all (with the exception of one clone whose fine structure is under investigation) were a part of a single 6 kb contiguous sequence which contained the previously described 1 kb DMD cDNA clone (cDNA5; Monaco et al., 1986). The six cDNA inserts that were detected with the 5' end probe all stopped within an estimated 100 bp interval (the five longest within a 50 bp interval). Concurrently, two mouse muscle cDNA libraries were being screened (Hoffman et al., 1987), and all the mouse DMD cDNAs obtained with 5' probes also ended within this same interval. This result indicated that the 5' end of the transcript may have been reached. Two additional "walks" were taken in the 3' direction using the most 3' segment of the existing

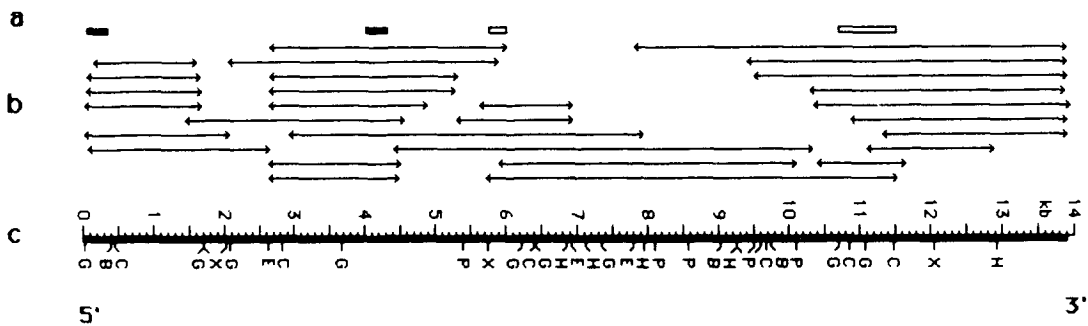
Cell  
510

Figure 1. Strategy of cDNA Cloning and Construction of the cDNA Restriction Map

From top to bottom: (a) black boxes represent the two probes used for the initial library screening; white boxes represent the probes used for the 2nd and 3rd library screening. (b) arrows represent the isolated cDNA inserts analyzed in this study. (c) cDNA restriction map. At least two restriction enzymes were tested for each insert presented in 1b and each restriction site presented on the map was observed on at least two independent cDNA clones. B = BamHI, C = HincII, E = EcoRI, G = BglII, H = HindIII, P = PstI, X = XbaI. Some additional XbaI sites may have been protected by methylation and may not have been detected.

cDNA as a hybridization probe against the library. The composite restriction map of the 14 kb of human cDNA obtained on these three screens is given in Figure 1, to-

gether with the screening strategy and the number of inserts analyzed. In the last walk against the human cDNA library, seven inserts out of nine analyzed stopped within



Figure 2. Nucleotide and Amino Acid Sequence Comparison of DMD cDNAs

Presented in the figure is the most 5' portion of the human and mouse cDNAs. For the human, the first 1.7 kb of the human DMD cDNA is presented and the first 824 bp is presented for the mouse. The human sequence is given above the mouse and differences between the mouse and human DNA sequences are indicated by asterisks on top of the human sequence, while amino acid differences are boxed. A minimum number of gaps was introduced in the first 200 nucleotides of either human or mouse sequences in order to maximize sequence alignment. Deduced amino acid sequences start at the putative methionine initiation codon of the DMD transcript. Sequence translations and alignments were done on the BIONET resource, Intelligenetics. The last 22 nucleotides of the human sequence (1678-1699) in the figure overlap with our other human sequence (Hoffman et al., 1987). The exact point where the overlap ends is indicated by an arrow. In addition, our previously presented mouse sequence (Hoffman et al., 1987) overlaps over the last 602 nucleotides at the 3' end of the human sequence presented here.

The Duchenne Muscular Dystrophy cDNA  
511

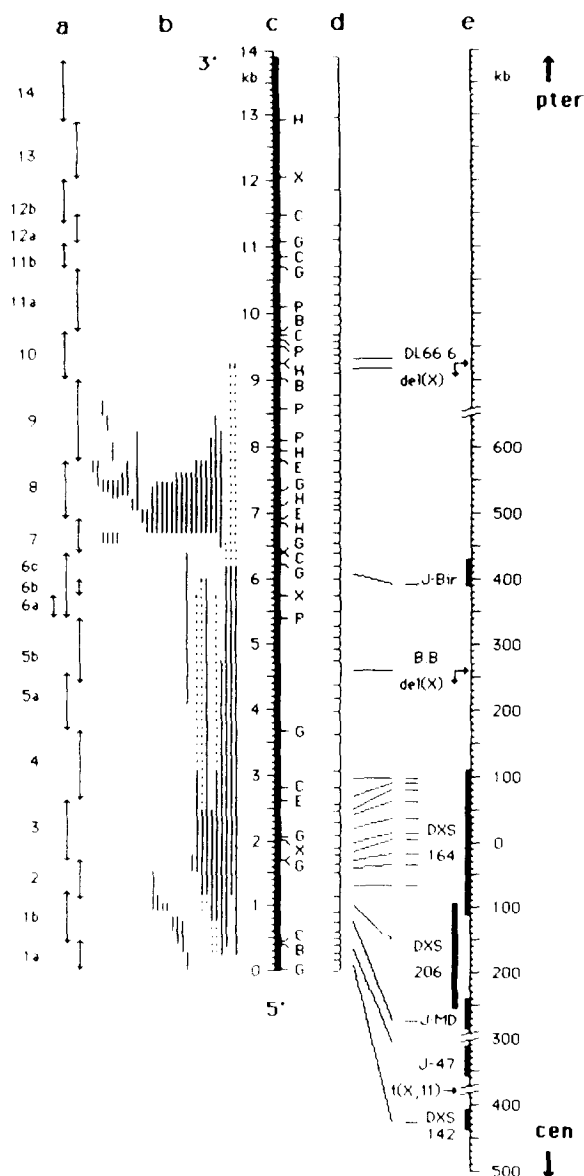


Figure 3. Schematic Representation of the DMD Locus and cDNA. From left to right: (a) cDNA fragments used as probes in this study (numbered according to their position along the cDNA map). (b) extension of 53 DMD deletions relative to the cDNA map; each line represents one deletion; dashed lines indicate uncertainty of the deletion breakpoint for deletions analyzed only with Xp21 genomic probes (analyzed prior to the cDNA cloning). (c) cDNA restriction map, as in Figure 1c. (d) brackets, portions of the cDNA detecting a single HindIII genomic fragment (the brackets are most of time only indicative). The sizes of the exon-containing HindIII genomic fragments are from bottom to top (in kilobases, parentheses indicate that the order is not known): (3.2; 3.25); 4.2; 8.5; 3.1; 8; 4.6; 7.5; 10.5; 4.2; 6.6; 2.7; 6; 1.7; 12; 3; 7.3; 11; 20; 5.2; (12; 4.7); 18; (0.45; 1.3; 1.8); 1.5; 6.6.2; (11; 4.2); (0.5; 4.1); 1.5; 10; (1.25; 3.8); 1.6; 3.7; 3.1; 7; (7.8; 1; 8.3); 2.3; (1; 8.8); 6; 3.5; (2.55; 2.8; 6.6; 12); 2.4; (1.45; 1.5; 1.9; 2.1; 5.2; 6.8); 10; 1.8; 5.9; 7.8; 6. When the HindIII fragment could be positioned relative to genomic loci it is indicated by a line to the genomic DNA map. The position of the third HindIII fragment (4.2 kb) distal to J-47 has been deduced by the analysis of this region in deletion DNA samples. Horizontal dashes indicate precise localization of coding sequences in cloned genomic sequences. (e) genomic physical map: black bars indicate cloned genomic sequences; arrows indicate translocation or deletion breakpoints; cen, the direction of the centromere and pter the direction of the short arm terminus. Scales are given for (c) and (e).

100 bp of each other, indicating that the 3' end of the transcript may also have been obtained.

Both the 5' and 3' extremes of the human cDNA, as well as the 5' end of the mouse cDNA, were subjected to DNA sequencing. Interspecies comparison of the sequences from the 5' end (Figure 2) showed that the first 200 bp are 80% homologous with multiple insertion/deletion differences and translation stop codons in all three reading frames. The first translation initiation codon (AUG) is conserved in both humans and mice; both sequences maintain a single open reading frame (ORF) exhibiting 92% homology at the DNA level and 96% homology at the protein level. This degree of homology is slightly greater than that observed for the next 2.1 kb of cDNA sequence (87% at both nucleotide and amino acid level; Hoffman et al., 1987). This region (Figure 2) of the DMD protein exhibits structural characteristics very similar to the 2.1 kb previously reported. The DNA sequence, together with the fact that the 5' terminus of all cDNA clones isolated to date (six human, ten mouse) end nearby each other, implies that the cloned cDNAs of both human and mouse contain at least part of the 5' untranslated sequence and presumably part of the 5' most exon. The sequences of the 3' end of two separate human cDNA inserts (data not shown) indicate that both have poly(A) tracks of 18 and 80 residues long, respectively, and each has the same polyadenylation signal (AATAAA; Proudfoot and Brownlee, 1976) from position -23 to -18 before the polyadenylation site, indicating that the 3' end of the transcript has also been reached. The last 22 bp of the primary sequence presented in Figure 2 overlaps with our other human sequence data (Hoffman et al., 1987) and the primary sequence presented in both papers completes the first 4.3 kb of sequence for the DMD transcript. The remaining 9.6 kb of sequence is currently being determined.

#### Genomic Mapping of the DMD Locus

The cDNA molecule was subdivided with restriction enzymes into approximately 1 kb fragments numerically designated according to their position along the cDNA map (Figure 3a). Each small fragment was used as a hybridization probe on Southern blots to demonstrate X chromosomal location and to begin the analysis for the genomic organization of the DMD gene. The Xp chromosomal origin of the cDNA sequences was shown by hybridization to HindIII-digested genomic DNA isolated from human cell lines with 49,XXXXY and 46,X i(Xq) karyotypes, and by hybridization to DNA isolated from a human-hamster cell line containing the X chromosome as the single human chromosome. In total, the 14 kb cDNA detects 65 different HindIII fragments, each showing a hybridization pattern consistent with Xp localization (an example is given in Figure 4D). Since there are five HindIII sites present in the cDNA sequence, the 14 kb DMD transcript is formed by a minimum of 60 exons (some HindIII genomic fragments may contain more than one exon). The human cDNA detected no fragments showing an Xq or an autosomal location. The order of the genomic fragments was deduced both from the order of cDNA subfrag-

Cell  
512

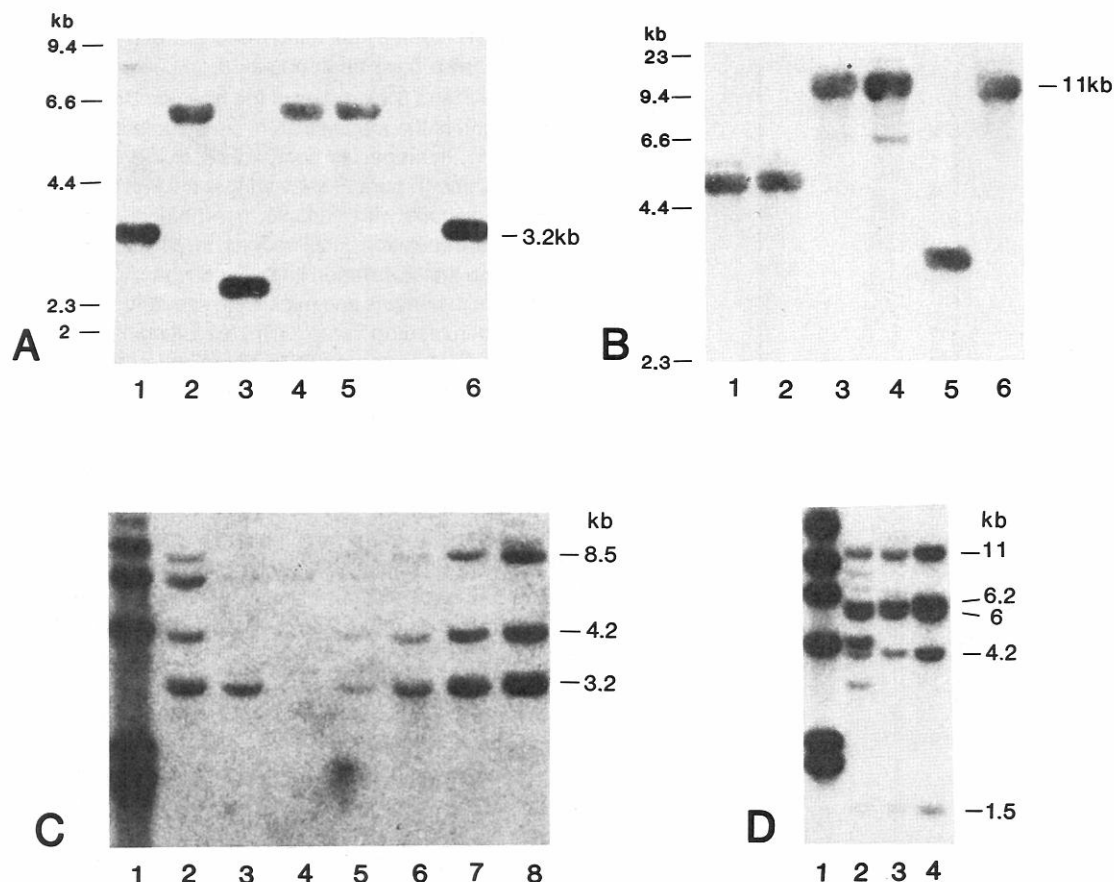


Figure 4. Genomic Mapping of Fragments Detected by cDNA Probes

(A) autoradiograph of probe 1a hybridized to the DNA of a phage obtained by "walking" from the DXS142 locus (unpublished data). The phage DNA was digested with the following enzymes: lane 1, HindIII/KpnI; 2, EcoRI/KpnI; 3, EcoRI/HindIII; 4, EcoRI; 5, EcoRI/SalI; 6, HindIII. (B) autoradiograph of probe 6c hybridized to the DNA of the phage W2-R of the J-Bir locus (Monaco et al., 1987): lane 1, EcoRI/SalI; 2, EcoRI; 3, HindIII/KpnI; 4, HindIII/SalI; 5, HindIII/EcoRI; 6, HindIII. (C) autoradiograph of probe 1a hybridized to HindIII cleaved DNA from the following sources: lane 2, human-hamster hybrid cell line G89 containing the X chromosome as single human chromosome; lane 3, hybrid cell line CF37 containing the Xp21→qter derivative chromosome of the X:11 translocation (Greenstein et al., 1977; Mohandas et al., 1980); lane 4, hybrid cell line 13R3 containing the Xpter→p21 derivative chromosome of the X:21 translocation (Verellen-Dumoulin et al., 1984; Worton et al., 1984); lane 5, hybrid cell line B12 containing the Xp21→qter derivative chromosome of the X:21 translocation; lane 6, normal male; lane 7, female with karyotype 46,X del(Xp21→qter) (Fryns et al., 1982; the del(X) chromosome is not deleted for the DXS164 and more centromeric loci); lane 8, human lymphoblastoid cell line GM1202 with 49,XXXXY karyotype. The 8.5 kb fragment in lane 5 (B12) was too faint to be reproduced on the printing. (D) autoradiograph of probe 6c hybridized to the following HindIII cleaved DNA: lane 2, human-hamster hybrid cell line G89 containing the X chromosome as single human chromosome; lane 3, human lymphoblastoid cell line LAZ463 with 46,X i(Xq) karyotype; lane 4, human lymphoblastoid cell line GM1202 with 49,XXXXY karyotype. HindIII-digested lambda DNA was used as a size marker (indicated on the left of Figures 4A and 4B and lane 1 in Figures 4C and 4D). Note that a common HindIII fragment is detected on both the respective phage and genomic Southern blot (3.2 kb with probe 1a and 11kb with probe 6c).

ments and from DMD deletions analysis (see below). The size and order (where known) of all detected HindIII fragments are given in the legend to Figure 3d. The last 2 kb of the cDNA (probes 13, 14, and part of 12), with an internal HindIII site, detect only two HindIII genomic fragments which are probably contiguous. The contiguous nature of these two HindIII fragments was demonstrated by the detection of a single restriction fragment upon hybridization of probes 13 and 14 to genomic DNA cleaved with 13 other restriction enzymes (data not shown). These data indicate that the last 2 kb of cDNA are probably encoded by a single exon.

The physical map of the DMD locus was analyzed first by mapping of hybridizing genomic HindIII fragments relative to well-characterized Xp21 translocation and deletion

breakpoints (Francke et al., 1985; Kunkel et al., 1985; Boyd et al., 1986; van Ommen et al., 1986). cDNA probes 1a to 6b were hybridized to the DNA isolated from the patient B.B., who has a large deletion in the Xp21 region (Francke et al., 1985). Beginning from the 5' end, the first 22 HindIII genomic fragments are absent from the genome of this patient, while the seven remaining fragments detected by the first 6 kb of cDNA are present. From the predicted orientation of transcription (Monaco et al., 1986) and the hybridization results, these latter 3' fragments must lie on the distal side of the deletion breakpoint. Moreover, the 23rd HindIII fragment is altered in size in this patient (10 kb instead of 15 kb), indicating either an extremely rare HindIII RFLP or that the deletion break occurred within this genomic fragment. cDNA probes 10 to

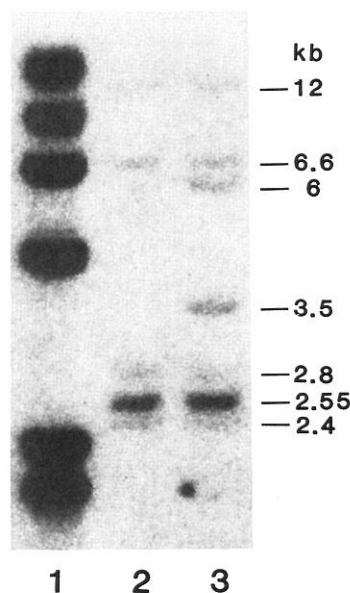


Figure 5. Mapping of the DL66.6 Deletion Breakpoint between Two DMD Exons

Autoradiograph of probe 10 hybridized to HindIII-digested DNA of patient DL66.6 (lane 2) and of a normal male (lane 3). Lane 1, HindIII-digested lambda DNA.

12b were hybridized to the DNA of the DMD patient DL66.6, who has a deletion with one break in the DXS164 locus and the other break distal from the J-Bir locus (van Ommen et al., 1986; Monaco et al., 1987). The distal breakpoint of DL66.6 occurs between two exons detected by probe 10 (Figure 5). As schematically depicted in Figure 3e, at least the last 4 kb of the cDNA is formed by exons lying distal to the DL66.6 deletion breakpoint.

cDNA probes 1a and 1b were tested against the t(X;21) and t(X;11) breakpoints known to be on the centromeric side of the DXS164 locus (the t(X;11) breakpoint being the most centromeric; Monaco et al., 1987). Two of the nine HindIII genomic fragments detected by these two cDNA probes are absent from the Xp21→qter derivative chromosome of both translocations (10.5 and 7.5 kb), five (4.6, 8, 3.1, 8.5, and 4.2 kb) are located between the two breakpoints, and one (3.2 kb) is present in the Xp21→qter derivative chromosome of both translocations (autoradiograph for probe 1a given in Figure 4C). This 3.2 kb HindIII fragment must be on the centromeric side of both translocation breakpoints. One HindIII fragment (3.25 kb) could not be localized because this weakly hybridizing fragment was obscured by the 3.2 kb band in this experiment.

In a second step, the genomic map was refined by testing for presence or absence of cDNA sequences in walked DNA surrounding various Xp21 cloned loci including DXS164 (pERT87), DXS142 (pERT84), J-Bir, J-MD, and J-47 (Monaco et al., 1987). In addition to the eight fragments described in Monaco et al. (1986), five new exon-containing fragments are present in our genomic walks surrounding these loci. Two additional fragments could be positioned within the DXS164 (pERT87) locus, one in the J-MD locus, and one in the J-Bir locus. Surprisingly, the 5'

most exon is in the DXS142 (pERT84) locus (the hybridization results for the latter two loci are shown in Figures 4A and 4B). In all cases, these results are consistent with the deletion/translocation mapping, especially the location of the first exon in DXS142 since this locus was previously shown to reside on the centromeric side of the t(X;11) breakpoint (Kunkel et al., 1985). No DMD coding sequences were found in the 50 kb of the J-47 locus. A schematic summary of all mapping results is given in Figure 3.

#### Analysis of the DMD Locus in Affected Individuals

Many deletions have previously been shown to disrupt the DMD locus in affected individuals (Kunkel et al., 1986). With exons of the cDNA spread over such a large region of Xp21, the various cDNA subclones provide ideal probes to search for deletions not detected by previously cloned loci. Among 104 DNA samples isolated from unrelated DMD boys that had been previously tested for deletion of Xp21 genomic clones, 94 could not be demonstrated to have a deletion. By testing these 94 samples with all cDNA subfragments, except probes 2, 3, 10, and 12a, 43 additional patient DNA samples were demonstrated to have deletions for parts of the DMD locus (example hybridizations for three cDNA subfragments are given in Figure 6). Including the ten previously detected deletions, the total number of observed deletions is 53 out of 104 samples. The DNA of 27 normal males was tested with the same cDNA probes; no deletions or HindIII fragment length polymorphisms were detected.

The incidence of deletions in DMD patients observed in our study of 104 DNA samples is a lower limit because not all cDNA subclones were a part of this initial analysis. In addition, deletions could have been missed because of the problem of comigrating fragments (see, for example, Figure 6B) or hybridizing fragments that are poorly visualized (presumably because the exons are very small). The extent and distribution of the deletions is shown in Figure 3b. Single exon deletions were rarely found. Even more remarkable is the observation that the deletions appear not to be evenly distributed along the DMD transcript. There is a small increase of deletions in the region detected with probe 1b and a large increase in the incidence of deletions in the region detected with probes 7 and 8. In addition, small deletions (relative to cDNA length) seem limited to these two regions. No deletions were found with probes 11a, 11b, 12b, 13, and 14.

In an effort to identify high frequency RFLPs useful in carrier detection and prenatal diagnosis, probes 1a, 4, 5a, 6b, 13, and 14 were hybridized to the DNA of four unrelated females that had been cleaved with 14 different restriction enzymes. No restriction fragment length polymorphisms were detected with probes 1a, 4, 13, and 14, while a BclI RFLP was detected with probe 5a and an XmnI polymorphism was detected with probe 6b (Figure 7). Probe 5a detects three constant fragments of 4.3, 6.2, and 7 kb and two allelic fragments of 19 kb ( $p = 0.51$ ) and 13 kb ( $q = 0.49$ ) on a BclI digest. Probe 6b detects two allelic XmnI fragments of 5.5 kb ( $p = 0.54$ ) and 4.7 kb ( $q = 0.46$ ). These two highly informative RFLPs lie near the middle of the

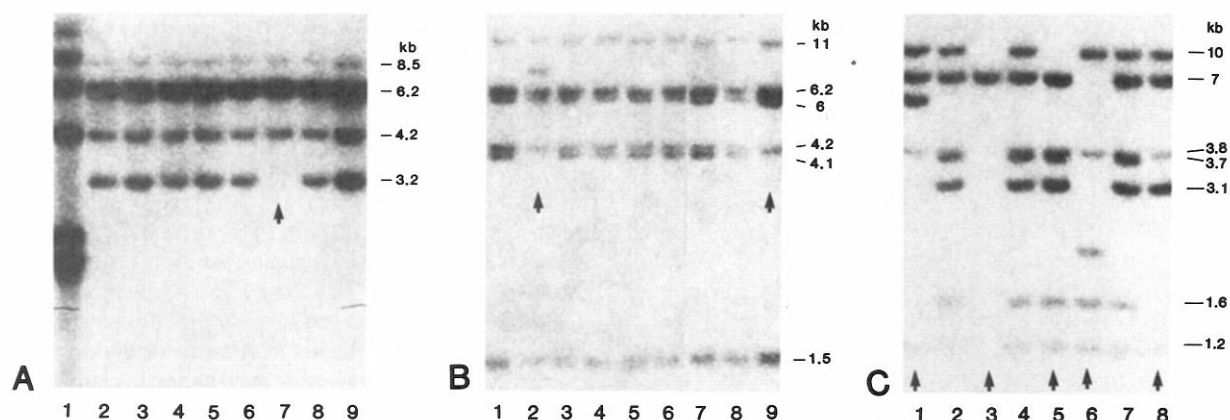
Cell  
514

Figure 6. Detection of Deletions in the DNA Isolated from DMD Patients Using cDNA Probes

(A) Autoradiograph of two probes 1a and 6b hybridized to HindIII-digested DNA isolated from DMD patients (lanes 2 to 9). Radiolabeled HindIII-digested lambda DNA was used as a size marker (lane 1). The fragments of size 3.2 kb (which is a doublet), 4.2 kb, and 8.5 kb are detected by probe 1a and the 6.2 kb fragment is detected by probe 6b (see Figure 3).

(B) Autoradiograph of two probes 6c and 7 hybridized to HindIII-digested DNA isolated from DMD patients (lanes 1 to 9). The 11 kb and 1.5 kb fragments are doublets (Figure 3) and a 0.5 kb fragment is not detected in this experiment. Hybridization of probe 7 alone to the DNA of patients 2 and 9 confirmed the deletions and showed that patient 2 also deletes the 10, 1.5, and 0.5 kb HindIII fragments corresponding to probe 7 (data not shown).

(C) Autoradiograph of probe 8 hybridized to HindIII-digested DNA isolated from DMD patients (lanes 1 to 8).

In each autoradiograph the patient DNA samples were different and the lanes demonstrating a deletion are indicated with an arrow.

DMD gene, and they will complement existing polymorphic probes in order to increase the accuracy of genetic diagnosis.

## Discussion

### Size and Features of the Normal DMD Gene

The complete cloning of the DMD cDNA has allowed for the initial analysis of the structure of the entire DMD gene. A minimum estimate of 60 exons form the 14 kb messenger RNA. Some of the coding segments are localized relative to previously mapped genomic segments. The mapping revealed that the 5' end of the gene lies in the DXS142 locus and the 3' end extends significantly further toward the telomere from the DL66.6 deletion breakpoint. In addition, a central exon of the transcript is encoded by the J-Bir locus.

The chromosome walking of DXS164 (pERT87), DXS206 (XJ1.1), and J-MD were shown to overlap by hybridization of end fragments of DXS164 and J-MD to the DXS206 walks (Burghes et al., 1987). From the determination of the relative order of the exon-containing HindIII fragments (see Figure 3d), a single 7.5 kb exon-containing fragment could be positioned in the DXS206 locus. Since the distance between J-MD and DXS164 is known from an Sfil-Sall fragment ending in the two loci (Burmeister et al., 1986) and from phage walking, the two introns flanking this fragment together must total more than 200 kb. Although this size is highly unusual for introns, the largest introns of the myosin heavy chain (MHC) gene are also found to reside in the 5' end of the gene (Strehler et al., 1986).

The J-Bir locus has been recently mapped as residing 300 kb from the end of the DXS164 locus with the B.B. de-

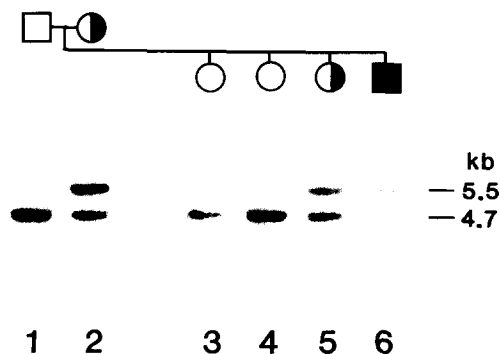


Figure 7. Segregation of the XmnI RFLP Detected with Probe 6b in a DMD Family

The symbols above each lane indicate the individual from the family whose DNA is separated by electrophoresis. The darkened regions indicate the DMD trait. The autoradiograph indicates that in this family the disease is segregating with the 5.5 kb allele of the RFLP.

letion breakpoint approximately halfway between (M. Burmeister, personal communication). The positioning of an exon within walked DNA of the J-Bir locus indicates that the first 6 kb of the transcript is encoded by exons spread over a minimum 800 kb of genomic DNA (see Figure 3e). The remaining 8 kb of the cDNA has not been mapped as precisely against the genomic map, mainly as a result of the lack of previously described reference points. The number of different hybridizing HindIII fragments for the 3' end remains similar to the 5' end of the gene and the entire locus is likely to approach 2000 kb in size. The DMD gene is actually the largest human gene yet identified, which presumably accounts for the high rate of new mutations and the high frequency of the disease in the human population (Moser, 1984).

**Incidence and Distribution of Deletion Mutations in the DMD Gene**

cDNA subfragments representing the majority of the DMD transcript were used as probes for the analysis of the DMD gene in affected boys. In a total of 104 DNA samples of unrelated DMD boys analyzed either with genomic or cDNA DMD probes, 50% exhibit deletions of part of the DMD gene. These deletions that encompass part of the coding sequences of the DMD gene are assumed to represent the primary DMD mutation since no DNA of normal males have been shown to be deletions. For diagnostic purposes, 27 deletions would have been detected with probe 8 and 15 others with probe 1b. These two probes cover less than 2 kb of the cDNA yet they detect nearly 80% of the deletions observed (42 out of 53). The proportion of DMD patients who can be demonstrated to have deletions is one of the highest yet observed. Steroid sulfatase deficiency (Ichthyosis), also located on the X chromosome short arm, has been shown to exhibit an even higher incidence of deletion (90%; Yen et al., 1987; Gillard et al., 1987; the authors suggest that the high rate of deletions may be due to the proximity of the STS gene with pseudoautosomal sequences of the X chromosome). In the case of DMD, it is tempting to correlate the high incidence of deletions with the large size of the gene. When the size of the locus is considered, then the overall incidence for deletion in any one 200 kb region (such as the DXS164 locus; Kunkel et al., 1986) is not unusually high compared to other loci (such as the HPRT locus; Yang et al., 1984; and the FVIIIc locus; Youssoufian et al., 1987).

Particularly intriguing is the striking number of deletion breakpoints found to originate within a single intron defined by probe 7 (Figure 3). One intron defined by exons contained in this probe exhibits 19 deletion breakpoints, or 36% of the total 53 deletions have one breakpoint in this region. Clearly this particular intron contains a characteristic that imparts a propensity for initiating deletion events. What might be unusual about this intron has yet to be determined, but the multiple breaks could be due to a sequence-specific rearrangement hot spot, or an extraordinarily large genomic distance spanned by this particular intron. The fact that no deletions were found with probes 11a, 11b, 12b, 13, and 14 might indicate that, in contrast to the latter region, distances spanned by these exons might be relatively small.

One of the most significant applications of molecular genetics to medicine to date has been the use of cloned DNA sequences to facilitate genetic studies of families at risk for a particular disease. Such studies have enabled the detection of carriers of certain diseases and the possibility of prenatal diagnosis (see, for example, Orkin and Kazazian, 1984). Since this type of family study employs RFLP analysis of many loci on numerous family members, the analyses are labor-intensive and expensive. Furthermore, the application of such studies to DMD families has proved less accurate than it has for many other hereditary diseases because of the relatively high frequency of meiotic recombination events between the specific mutation giving rise to the disease and the flanking markers

employed (Darras et al., 1987; Kunkel et al., 1986; Fischbeck et al., 1986), which is undoubtedly the result of the extremely large size of the DMD gene. We have found that at least 50% of affected individuals exhibit easily detectable deletions. Prediction of individuals at risk for DMD in a family where a deletion has been detected can be performed accurately and quickly. In such "deletion families," meiotic recombination events would become irrelevant to the diagnosis of carriers and affected individuals since one would be looking at the site of mutation rather than cosegregating flanking markers. The routine analysis of families at risk for DMD using the complete DMD cDNA could dramatically decrease the cost and labor involved and greatly increase the accuracy of such studies.

The 50% of families that do not exhibit a deletion but still carry a DMD mutation must be analyzed by more traditional linkage analyses. The identification of the 5' and 3' ends of the DMD gene will direct the search for RFLPs in these regions, such that intragenic recombinants can be detected. These gene flanking RFLPs in combination with the new ones described here and other RFLPs (Goodfellow et al., 1985) should greatly increase the accuracy of prenatal diagnosis and carrier detection in nondeletion families.

**Conclusion**

Deletion of part or all of the DMD locus is one of the major causes of DMD in the human population. These deletions are accurately and easily detected with cDNA subfragments. The DMD cDNA is encoded by exons spread over approximately two million base pairs of the human X chromosome short arm. The genomic locus is nearly 200 times greater in size than the final RNA transcript, with a mean size for exons of 200 bp and a mean size for introns of 35 kb. The DNA encoding the DMD locus represents nearly 1/1000th of total human DNA and is close to one-third the amount of the entire *E. coli* genome. The high frequency of DMD mutations in the human population may be a direct consequence of this extremely large size. The question remains as to why the gene is so large. The answers should be forthcoming as the remainder of the region is fully characterized.

**Experimental Procedures****Construction of the cDNA Library**

The poly(A)<sup>+</sup> RNA used for the construction of the cDNA library was prepared from the psoas muscle of a 20-22 week old human female fetus and was tested for the presence of the 16 kb DMD transcript. RNA isolation and Northern blotting was as in Monaco et al. (1986). The library was constructed using a modification of the procedure of Gubler and Hoffman (1983) following the modifications provided by Stratagene's librarian, Ms. Heidi Short (the detailed procedure is available upon request). Five micrograms of poly(A)<sup>+</sup> RNA was used for oligo(dT) priming and first strand synthesis with AMV reverse transcriptase. The second strand was synthesized with *E. coli* DNA polymerase I using RNAase H for the creation of RNA primers. The cDNAs were incubated successively with T4 DNA polymerase, RNAase A, polynucleotide kinase, and AMV reverse transcriptase. The cDNAs internal EcoRI sites were methylated and EcoRI linkers (Biolabs) were ligated to the cDNAs and subsequently EcoRI restricted. cDNAs were size-

fractionated on a Sepharose 4B exclusion column. Two hundred and fifty nanograms of cDNA of the first fractions were ligated to 5 µg of lambda gt10 EcoRI cut DNA (Huynh et al. 1985; Stratagene), of which 1/20th was packaged with the Gigapack Gold kit (Stratagene) yielding  $1.8 \times 10^6$  phages.

#### Screening of the cDNA Library

The library was plated and screened according to Benton and Davis (1977). Two mouse muscle cDNA libraries were constructed prior to the construction of the human cDNA library. Hybridization of these mouse libraries with the 1 kb human DMD cDNA (cDNA 5; Monaco et al., 1986) resulted in the acquisition of 4.3 kb of mouse cDNA (Hoffman et al., submitted). For the first "walk" against the human library, the 5' and 3' end fragments (black boxes, Figure 1a) of the 4.3 kb mouse cDNA were hybridized to approximately  $1.1 \times 10^6$  primary unamplified phages immobilized on nitrocellulose filters. The same filters were reused for the second and third "walks." The cDNA inserts were subcloned in the EcoRI site of the bluescribe or bluescript plasmid vector (Stratagene).

#### cDNA Sequencing

The human sequences were determined by the dideoxynucleotide method (Sanger et al., 1977). For the 5' sequences, two independent cDNA clones were deleted, each in one direction by the technique of Yanisch-Perron et al. (1985) using the "Exo/Mung" kit (Stratagene) to create appropriate templates for the determination of the sequence for both strands. The 3' sequence was determined from the 3' end of three independent cDNA clones, one of which ended two nucleotides before the polyadenylation site. The mouse sequences were determined either on both strands or three times on the same strand (nucleotides 500 to 824) by either the Maxam and Gilbert method (1980) or the dideoxynucleotide method (Sanger et al., 1977).

#### Southern Blotting and Cell Lines

Preparation of high molecular weight genomic DNA from whole blood leukocytes of patients or established fibroblast and lymphoblastoid cell lines, restriction endonuclease digestion, agarose gel electrophoresis, Southern blotting, and DNA probe labeling and hybridization conditions are as previously described (Aldridge et al., 1984; Monaco et al., 1985).

The lymphoblastoid cell lines LCL119 (derived from the B.B. deletion patient; Francke et al., 1985) and LAZ463 (with a 46,Xi(Xq) karyotype; Kunkel et al., 1982) were provided by Dr. H. Ochs (Seattle) and Dr. S. Latt (Boston), respectively. The lymphoblastoid cell line GM1202 (with a 49,XXXXY karyotype) was obtained from the Human Genetic Mutant Repository (Camden, NJ). The human-rodent hybrid cell lines G89 (containing the X chromosome as single human chromosome), CF37 (containing the Xp21-qter derivative chromosome of the X:11 translocation; Greenstein et al., 1977; Mohandas et al., 1980), 13R3 and B12 (containing, respectively, the Xpter-p21 and Xp21-qter derivative chromosome of the X:21 translocation; Verellen-Dumoulin et al., 1984; Worton et al., 1984) were provided by Dr. G. P. A. Bruns (Boston), Dr. T. Mohandas (Los Angeles), and Dr. I. Craig (London), respectively. DNA from the DL66.6 deletion patient was provided by Dr. G. J. B. van Ommen (Leiden). The panel of 104 independent DMD boys was constituted from four different sources. Blood samples of 29 DMD boys were collected here at the Children's Hospital (Boston). DNA samples of 37, 16, and 22 DMD boys were kindly provided by Dr. R. Worton (Toronto), Dr. K. H. Fischbeck (Philadelphia), and Dr. M. J. Denton (Sidney), respectively. Deletions in the DXS164 locus were found for ten of these boys (Monaco et al., 1985; Kunkel et al., 1986) and the extent of the deletions has been analyzed (Monaco et al., 1987 and unpublished results).

#### Acknowledgments

We gratefully acknowledge the contribution of patient DNA samples from Drs. Kenneth H. Fischbeck, Michael J. Denton, and Ronald Worton. We also thank the various investigators who have sent us cell lines which helped in the mapping of the DMD gene. We thank Dr. Samuel Latt for critical reading of the manuscript. L. M. K. is an associate investigator of the Howard Hughes Medical Institute; A. P. M. is supported in part by PHS NRSA (2T 32 GM07753-07) from the National Institute

for General Medical Sciences; M. K., E. P. H., A. P. M. receive support from the Muscular Dystrophy Association of America. This work was supported in part by the National Institutes of Health (#HD 18658 and NS23740) and by the Muscular Dystrophy Association of America.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 30, 1987.

#### References

- Aldridge, J., Kunkel, L., Bruns, G., Tantravahi, U., Lalande, M., Brewster, T., Moreau, E., Wilson, M., Bromley, W., Roderick, T., and Latt, S. (1984). A strategy to reveal high-frequency RFLPs along the human X chromosome. *Am. J. Hum. Genet.* 36, 546-564.
- Bakker, E., Hofker, M. H., Goor, N., Mandel, J. -L., Wrogemann, K., Davies, K. E., Kunkel, L. M., Willard, H. F., Fenton, W. A., Sandkuyil, L., Majoor-Krakauer, D., Van Essen, A. J., Jahoda, M. G. J., Sachs, E., van Ommen, G. J. B., and Pearson, P. L. (1985). Prenatal diagnosis and carrier detection of Duchenne muscular dystrophy with closely linked RFLPs. *Lancet* i, 655-658.
- Benton, W. D., and Davis, R. W. (1977). Screening lambda gt recombinants clones by hybridization to single plaques in situ. *Science* 196, 180-192.
- Boyd, Y., Buckle, V., Holt, S., Munroe, E., Hunter, D., and Craig, I. (1986). Muscular dystrophy in girls with X-autosome translocations. *J. Med. Genet.* 23, 484-490.
- Burghes, A. H. M., Logan, C., Hu, X., Belfall, B., Worton, R., and Ray, P. N. (1987). Isolation of a cDNA clone from the region of an X:21 translocation that breaks within the Duchenne/Becker muscular dystrophy gene. *Nature*, in press.
- Burmeister, M., and Lehrach, H. (1986). Long-range restriction map around the Duchenne muscular dystrophy gene. *Nature* 324, 582-585.
- Darras, B. T., Harper, J. F., and Francke, U. (1987). Prenatal diagnosis and detection of carriers with DNA probes in Duchenne's muscular dystrophy. *New Eng. J. Med.* 316, 985-992.
- Davies, K. E., Pearson, P. L., Harper, P. S., Murray, J. M., O'Brien, T., Sarfarazi, M., and Williamson, R. (1983). Linkage analysis of two cloned DNA sequences flanking the Duchenne muscular dystrophy locus on the short arm of the human X chromosome. *Nucl. Acids Res.* 11, 2303-2312.
- Fischbeck, K. H., Ritter, A. W., Tirschwell, D. L., Kunkel, L. M., Bertelson, C. J., Monaco, A. P., Hejtmancik, J. F., Boehm, C., Ionasescu, V., Ionasescu, R., Pericak-Vance, M., Kandt, R., and Roses, A. D. (1986). Recombination with pERT87 (DXS164) in families with X-linked muscular dystrophy. *Lancet* ii, 104.
- Francke, U., Ochs, H. D., de Martinville, B., Giacalone, J., Lindgren, V., Distèche, C., Pagon, R. A., Hofker, M. H., van Ommen, G. J. B., Pearson, P. L., and Wedgwood, R. J. (1985). Minor Xp21 chromosome deletion in a male associated with expression of Duchenne muscular dystrophy, chronic granulomatous disease, retinitis pigmentosa and McLeod syndrome. *Am. J. Hum. Genet.* 37, 250-267.
- Fryns, J. P., Kleczkowska, A., Petit, P., and van den Berghe, H. (1982). Fertility in patients with X-chromosome deletions. *Clin. Genet.* 22, 76-79.
- Gillard, E. F., Affara, N. A., Yates, J. R. W., Goudie, D. R., Lambert, J., Aiken, D. A., and Ferguson-Smith, M. A. (1987). Deletion of a DNA sequence in eight of nine families with X-linked ichthyosis (steroid sulfatase deficiency). *Nucl. Acids. Res.* 15, 3977-3985.
- Goodfellow, P. N., Davies, K. E., and Ropers, H. H. (1985). Report of the committee on the genetic constitution of the X and Y chromosomes. *Human Gene Mapping 8. Cytogenet. Cell Genet.* 40, 296-352.
- Greenstein, R. M., Reardon, M. P., and Chan, T. S. (1977). An X-autosome translocation in a girl with Duchenne muscular dystrophy, evidence for DMD gene localisation. *Pediatr. Res.* 11, 475A.
- Gubler, U., and Hoffman, B. (1983). A simple and very efficient method for generating cDNA libraries. *Gene* 25, 263-269.
- Hoffman, E. P., Monaco, A. P., Feener, C., and Kunkel, L. M. (1987).

The Duchenne Muscular Dystrophy cDNA  
517

Conservation of the Duchenne muscular dystrophy gene in mice and humans. *Science*, in press.

Huynh, T., Young, R., and Davis, R. W. (1985). Constructing and screening cDNA libraries in lambda gt10 and gt11. In *DNA Cloning*, Volume 1, D. M. Glover, ed. (Washington, D.C.: IRL Press), pp. 49–78.

Kenwick, S., Patterson, M., Speer, A., Fischbeck, K., and Davies, K. (1987). Molecular analysis of the Duchenne muscular dystrophy region using pulsed field gel electrophoresis. *Cell* 48, 351–357.

Kingston, H. M., Harper, P. S., Pearson, P. L., Davies, K. E., Williamson, R., and Page, D. (1983). Localization of the gene for Becker muscular dystrophy. *Lancet* ii, 1200.

Kunkel, L. M., Tantravahi, U., Eisenhard, M., and Latt, S. A. (1982). Regional localization on the human X of DNA segments cloned from flow sorted chromosomes. *Nucl. Acids Res.* 10, 1557–1578.

Kunkel, L. M., Monaco, A. P., Middlesworth, W., Ochs, H., and Latt, S. A. (1985). Specific cloning of DNA fragments absent from the DNA of a male patient with an X-chromosome deletion. *Proc. Natl. Acad. Sci. USA* 82, 4778–4782.

Kunkel, L. M., et al. (1986). Analysis of deletions in DNA of patients with Becker and Duchenne muscular dystrophy. *Nature* 322, 73–77.

Maxam, A. M., and Gilbert, W. (1980). Sequencing end-labeled DNA with base-specific chemical cleavages. *Meth. Enzymol.* 65, 499–560.

Mohandas, T., Sparkes, R. S., Hellkuhl, B., Grzeschik, K. H., and Shapiro, L. J. (1980). Expression of an X-linked gene from an inactive human X chromosome in mouse-human hybrid cells: further evidence for the non-inactivation of the steroid sulfatase locus in man. *Proc. Natl. Acad. Sci. USA* 77, 6759–6763.

Monaco, A. P., and Kunkel, L. M. (1987). A giant locus for the Duchenne and Becker muscular dystrophy gene. *TIG* 3, 33–37.

Monaco, A. P., Bertelson, C. J., Middlesworth, W., Colletti, C.-A., Aldridge, J., Fischbeck, K. H., Bartlett, R., Pericak-Vance, M. A., Roses, A. D., and Kunkel, L. M. (1985). Detection of deletions spanning the Duchenne muscular dystrophy locus using a tightly linked DNA segment. *Nature* 316, 842–845.

Monaco, A. P., Neve, R. L., Colletti-Feener, C., Bertelson, C. J., Kurnit, D. M., and Kunkel, L. M. (1986). Isolation of candidate cDNAs for portions of the Duchenne muscular dystrophy gene. *Nature* 323, 646–650.

Monaco, A. P., Bertelson, C. J., Colletti-Feener, C., and Kunkel, L. M. (1987). Localization and cloning of Xp21 deletion breakpoints involved in muscular dystrophy. *Hum. Genet.* 75, 221–227.

Moser, H. (1984). Duchenne muscular dystrophy: pathogenic aspects and genetic prevention. *Hum. Genet.* 66, 17–40.

Orkin, S. H., and Kazazian, H. H., Jr. (1984). Mutation and polymorphism of the human beta-globin gene and its surrounding DNA. *Ann. Rev. Genet.* 18, 131–171.

Proudfoot, N. J., and Brownlee, G. G. (1976). 3' non-coding region sequences in eukaryotic mRNA. *Nature* 263, 211–214.

Ray, P. N., Belfall, B., Duff, C., Logan, C., Kean, V., Thompson, M., W., Sylvester, J. E., Gorski, J. L., Schmickel, R. D., and Worton, R. G. (1985). Cloning of the breakpoint of an X:21 translocation associated with Duchenne muscular dystrophy. *Nature* 318, 672–675.

Rozen, R., Fox, J., Fenton, W. A., Horwich, A. L., and Rosenberg, L. E. (1985). Gene deletion and restriction fragment length polymorphisms at the human ornithine transcarbamylase locus. *Nature* 313, 815–817.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74, 5463–5467.

Strehler, E. E., Strehler-Page, M.-A., Perriard, J.-C., Periasamy, M., and Nadal-Ginard, B. (1986). Complete nucleotide and encoded amino acid sequence of a mammalian myosin heavy chain gene. *J. Mol. Biol.* 190, 291–317.

van Ommen, G. J. B., Verkerk, J. M. H., Hofker, M. H., Monaco, A. P., Kunkel, L. M., Ray, P., Worton, R., Wieringa, B., Bakker, E., and Pearson, P. L. (1986). A physical map of 4 million bp around the Duchenne muscular dystrophy gene on the human X chromosome. *Cell* 47, 499–504.

Verellen-Dumoulin, C., Freund, M., de Meyer, R., Laterre, C., Frederic,

J., Thompson, M. W., Markovic, V. D., and Worton, R. G. (1984). Expression of an X-linked muscular dystrophy in a female due to translocation involving Xp21 and non-random X-inactivation. *Hum. Genet.* 67, 115–119.

Worton, R. G., Duff, C., Sylvester, J. E., Schmickel, R. D., and Willard, H. F. (1984). Duchenne muscular dystrophy involving translocation of the DMD gene next to ribosomal DNA genes. *Science* 224, 1447–1448.

Yang, T. P., Patel, P. I., Craig Chinault, A., Stout, J. T., Jackson, L. G., Hildebrand, B. M., and Caskey, T. (1984). Molecular evidence for new mutation at the HPRT locus in Lesch-Nyhan patients. *Nature* 310, 412–414.

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and PUC19 vectors. *Gene* 33, 103–119.

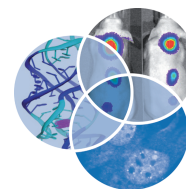
Yen, P., Allen, E., Marsh, B., Mohandas, T., Wang, N., Taggart, R. T., and Shapiro, L. (1987). Cloning and expression of steroid sulfatase cDNA and the frequent occurrence of deletions of STS deficiency: implications for X-Y interchange. *Cell* 49, 443–454.

Youssoufian, H., Antonarakis, S., Aronis, S., Tsiftis, G., Phillips, D., and Kazazian, H. H., Jr. (1987). Characterization of five partial deletions of the factor VIII gene. *Proc. Natl. Acad. Sci. USA* 84, 3772–3776.

Zatz, M., Vianna-Morgante, A. M., Campos, P., and Diamant, A. J. (1981). Translocation (X:6) in a female with Duchenne muscular dystrophy, implications for the localisation of the DMD locus. *J. Med. Genet.* 18, 442–447.

# EXHIBIT 48

## Advanced Review



# Targeting RNA splicing for disease therapy

Mallory A. Havens,<sup>1</sup> Dominik M. Duelli<sup>2</sup> and Michelle L. Hastings<sup>1\*</sup>

Splicing of pre-messenger RNA into mature messenger RNA is an essential step for the expression of most genes in higher eukaryotes. Defects in this process typically affect cellular function and can have pathological consequences. Many human genetic diseases are caused by mutations that cause splicing defects. Furthermore, a number of diseases are associated with splicing defects that are not attributed to overt mutations. Targeting splicing directly to correct disease-associated aberrant splicing is a logical approach to therapy. Splicing is a favorable intervention point for disease therapeutics, because it is an early step in gene expression and does not alter the genome. Significant advances have been made in the development of approaches to manipulate splicing for therapy. Splicing can be manipulated with a number of tools including antisense oligonucleotides, modified small nuclear RNAs (snRNAs), *trans*-splicing, and small molecule compounds, all of which have been used to increase specific alternatively spliced isoforms or to correct aberrant gene expression resulting from gene mutations that alter splicing. Here we describe clinically relevant splicing defects in disease states, the current tools used to target and alter splicing, specific mutations and diseases that are being targeted using splice-modulating approaches, and emerging therapeutics. © 2013 John Wiley & Sons, Ltd.

## How to cite this article:

*WIREs RNA* 2013, 4:247–266. doi: 10.1002/wrna.1158

## INTRODUCTION

**P**re-mRNA (messenger RNA) splicing is the process of removing introns from pre-mRNA and ligating together exons to produce a mature mRNA that represents the template for protein translation. Any splicing errors will result in a disconnection between the coding gene and its encoded protein product. The splicing reaction must occur with high efficiency and fidelity in order to maximize gene expression and avoid the production of aberrant proteins.<sup>1</sup> A complex macromolecular machine, termed the spliceosome, catalyzes this reaction. The spliceosome consists of a dynamic set of hundreds of proteins and small RNAs.<sup>2</sup> The complexity of the spliceosome is likely

key to achieving the splicing specificity of the diverse set of sequences that define exons and introns.<sup>1</sup>

The most conserved sequences that define exons and introns are the core splice site elements comprised of the 5' splice site (5'ss), the branchpoint sequence (BPS), the polypyrimidine (Py) tract, and the 3' splice site (3'ss) (Figure 1(a)). These intronic sequences demarcate exons and are recognized, in most splicing reactions, by specific base-pairing interactions with the small nuclear RNA (snRNA) components of five ribonucleoproteins (snRNPs), U1, U2, U4, U5, and U6.<sup>2</sup> These snRNPs are essential for orchestrating the splicing reaction, which occurs between bases within the core splicing sequences. The splicing reaction is initiated by U1 snRNP binding to the 5'ss, followed by U2 snRNP interactions at the BPS and finally U4, U5, and U6 snRNP interactions near the 5' and 3' splice sites. The spliceosome is also made up a large number of other splicing factors, in addition to the snRNPs, including RNA binding proteins which bind in a sequence-specific manner to RNA and either enhance or silence splicing at nearby splice sites.<sup>1</sup> These so-called splicing enhancers and silencers can

\*Correspondence to: Michelle.Hastings@rosalindfranklin.edu

<sup>1</sup>Department of Cell Biology and Anatomy, Chicago Medical School, Rosalind Franklin University of Medicine and Science, North Chicago, IL, USA

<sup>2</sup>Department of Cellular and Molecular Pharmacology, Chicago Medical School, Rosalind Franklin University of Medicine and Science, North Chicago, IL, USA

The authors have declared no conflicts of interest for this article.

be classified by their locations in either exons or introns (ESE and ISE, for exonic or intronic splicing enhancers, and ESS and ISS for exonic or intronic splicing silencers, respectively (Figure 1(a)).<sup>1</sup>

Most pre-mRNAs can be spliced in different ways to produce distinct mature mRNA isoforms in a process called alternative splicing. Alternative splicing most commonly involves skipping of an exon(s) (Figure 1(a)), although the use of different 5'ss and 3'ss are also common alternative splicing events. In many cases, the distinct mRNA isoforms produced from alternative splicing will code for proteins that have anywhere from subtle to dramatic functional differences.<sup>3</sup> Alternative splicing is an important mechanism to generate the phenotypic diversity of higher eukaryotes in that it expands gene expression complexity without an increase in the overall number of genes.<sup>3</sup> Alternative splicing is estimated to occur in most pre-mRNAs,<sup>3</sup> suggesting that splicing of most gene transcripts has an inherent flexibility that promotes modulation of protein expression and activity. Indeed, flanking introns often measure in multiples of the tri-nucleotide code, such that skipping a particular exon maintains the reading frame in the resulting mRNA. This mRNA then codes for a protein with an internal deletion corresponding to the loss of the amino acids encoded by the skipped exon.<sup>4</sup> Developmental, as well as tissue- and cell-type specific alternative splicing is common, with variations in the relative abundance of different spliced isoforms, which suggests a dynamic regulation.<sup>5</sup>

This review summarizes the different types of disease-causing genetic mutations that can affect splicing, and discusses other forms of splicing deregulation that are associated with disease. Different approaches that are currently investigated as candidate therapeutics to alter splicing as a means to compensate for or to correct aberrant splicing are highlighted.

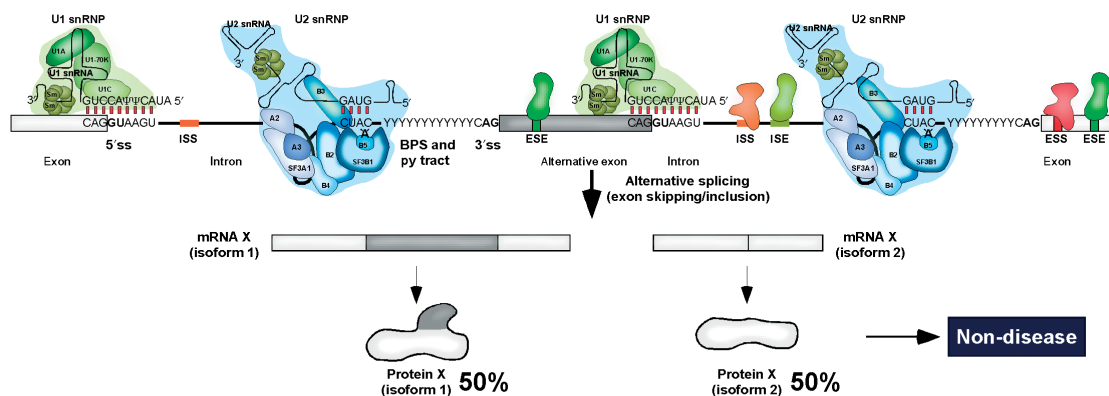
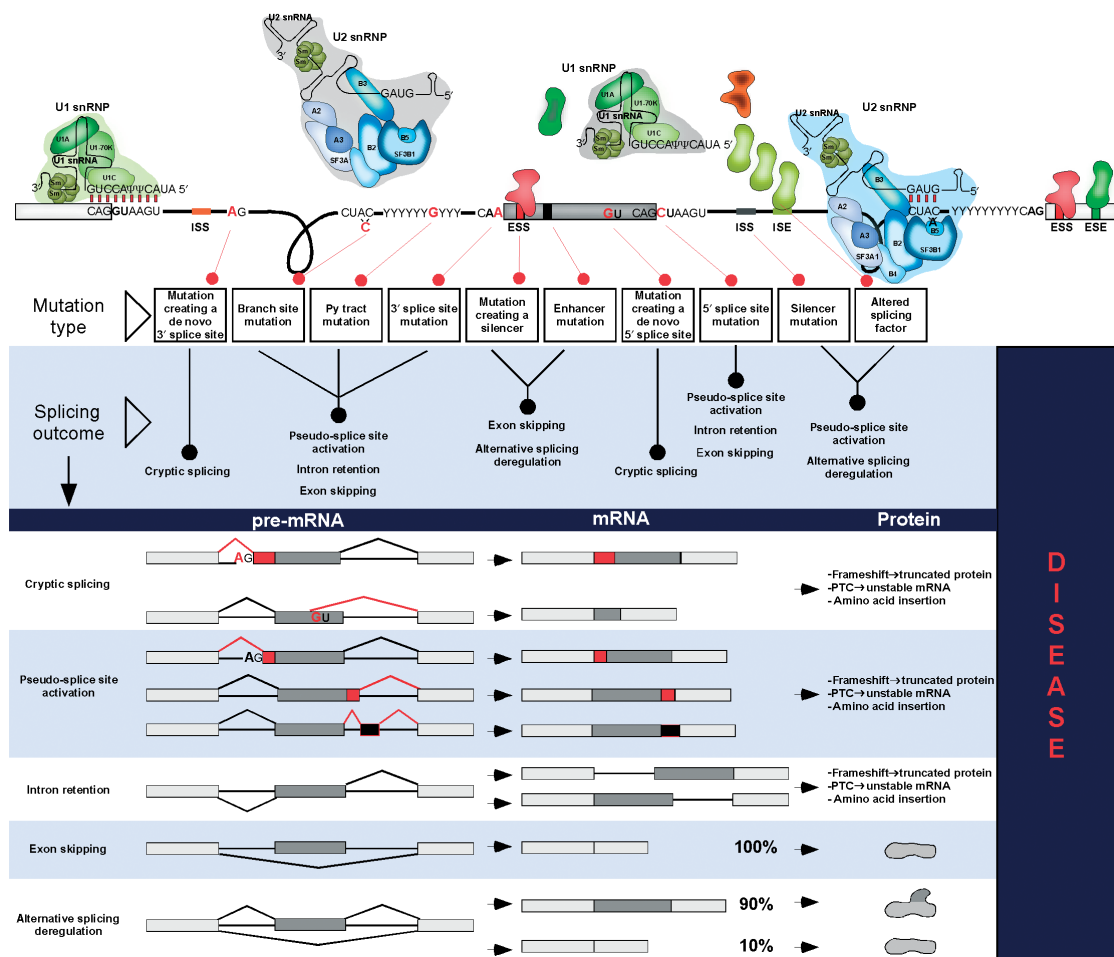
## SPlicing DEFECTS THAT CAUSE DISEASE: THE PROBLEMS

Mutations that disrupt normal splicing have been estimated to account for up to a third of all disease-causing mutations.<sup>6,7</sup> Although diseases and conditions caused by mutations that disrupt normal pre-mRNA splicing are common, it can be difficult to identify a splicing defect as the cause of a disease. Disease-associated mutations that occur within introns are, by default, usually assumed to alter splicing patterns, because they do not alter the coding sequence. Intronic mutations may disrupt the core splice sites (sequences within the 5'ss or 3'ss, the

Py tract or BPS). Such mutations typically result in the skipping of the exon(s) upstream or downstream of the mutated splice site or in the retention of the intron (Figure 1(b), lower panel). In many cases, when the authentic splice site is mutated a pseudo splice site (a weak consensus splice site that is not recognized as a splice site under normal conditions) is activated within the flanking exon or intron<sup>8</sup> (Figure 1(b)). Mutations in introns or exons can also disrupt or create *de novo* splicing silencers and enhancers or *de novo* cryptic splice sites (splice sites that are created by mutations). These types of mutations can affect splicing in a similar manner as mutations in the consensus splice sites and can also result in deregulation of alternative splicing (Figure 1). Intronic splice site mutations account for approximately 10–15% of annotated disease mutations.<sup>9</sup>

Mutations within coding exons can also affect splicing. Exonic mutations can result in the creation of a *de novo* cryptic splice site, disruption of an RNA secondary structure that has a regulatory function, or they can disrupt a splicing silencer or enhancer rendering the site unrecognizable by the sequence-specific RNA-binding protein that is required for splicing at a particular site. Such exonic mutations can be silent mutations or missense mutations, and thus may or may not alter the coding sequence. If a mutation changes coding, the molecular basis for the disease may be mistakenly ascribed to the change in amino acid incorporation rather than to aberrant splicing. Computational analysis of these types of exonic mutations predict that as many as 25% of mutations within exons alter splicing.<sup>6,7</sup> Thus, overall, when considering both exonic and intronic locations, more than 30% of known genetic mutations are predicted to alter splicing.<sup>6</sup> The outcome of splicing mutations is usually the formation of an aberrant mRNA or quantitative changes in alternative mRNA isoform abundance and consequently loss of normal protein expression (Figure 1(b)). Other causes of aberrant constitutive or alternative splicing include mutations or quantitative changes of a protein that regulates splicing (Figure 1(b)). In this situation, aberrant splicing can occur in all of the RNA transcripts that are processed by the affected protein.

Rather than correcting aberrant splicing, the splicing reaction can also be targeted to induce aberrant splicing as a way to disrupt gene expression of proteins involved in disease pathogenesis. Similarly, splicing can be targeted to cause the skipping of exons that have nonsense mutations or deletions that disrupt protein coding. Such skipping of exons can be used to reframe and rescue protein expression. Nonsense mutations account for more than 10% of annotated

**(a) Pre-mRNA splicing and alternative splicing****(b) Splicing mutations**

**FIGURE 1** | Splicing, alternative splicing, and pathogenic mutations that affect splicing outcomes. (a) A model of the splicing sequences and the components involved in their initial recognition during splicing by the major spliceosome. Exons are depicted as boxes and introns are lines. The canonical 5' splice site (5'ss), branch point sequence (BPS), polypyrimidine tract (py tract), and 3' splice site (3'ss) sequences are shown along with their interactions with the U1 and U2 snRNPs. The gray-lined snRNA and the major protein components of the snRNPs are labeled. Intronic and exonic splicing silencers (orange: ISS and red: ESS) and enhancers (dark green: ESE and light green: ISE) are depicted either with or without their *trans*-acting proteins bound. Alternative splicing of the middle exon produces mRNA isoforms 1 and 2 and results in two distinct protein isoforms, 1 and 2. (b) Common types of disease-causing mutations that disrupt splicing are labeled in red along with their possible outcomes and the aberrant splicing pathway (bottom). *De novo* cryptic splice site mutations are represented by the terminal dinucleotides, GU (5'ss) and AG (3'ss).

disease-causing mutations.<sup>9</sup> Even more prevalent are small deletions and insertions, which account for more than 20% of documented mutations.<sup>9</sup> Given the prevalence of this type of mutation, the approach of splicing induced reading frame correction or reframing has potential to have a significant impact for disease therapeutics.

Whether as a tool to correct aberrant splicing or to induce aberrant splicing, the opportunities and impacts of therapeutics that target splicing are vast and a number of strategies have been developed to successfully treat disease by RNA splicing intervention.

## MANIPULATING SPLICING: THE TOOLS

Targeting RNA splicing to correct the effects of a mutation bypasses the need to correct or replace mutated DNA or diseased cells, which is the approach of two other major therapeutic platforms: gene-replacement and some stem-cell therapies. Emerging RNA splicing-targeted therapies are proving to be a promising and powerful therapeutic approach, in part because of the wide range of mutations that can be corrected, the ease of delivery, and the success of the approaches in treating disease. The following section describes some of the basic tools that have been developed to manipulate splicing. For each approach, there have been numerous types of modifications developed to improve therapeutic and delivery methods. These improvements have helped the success of manipulating splicing as a therapy,<sup>10</sup> but will not be discussed in detail here. The intent of this review is to outline the general functional utility of the tools that have been developed and to provide an overview of specific examples of how these tools have been used to modulate splicing for potential disease therapy.

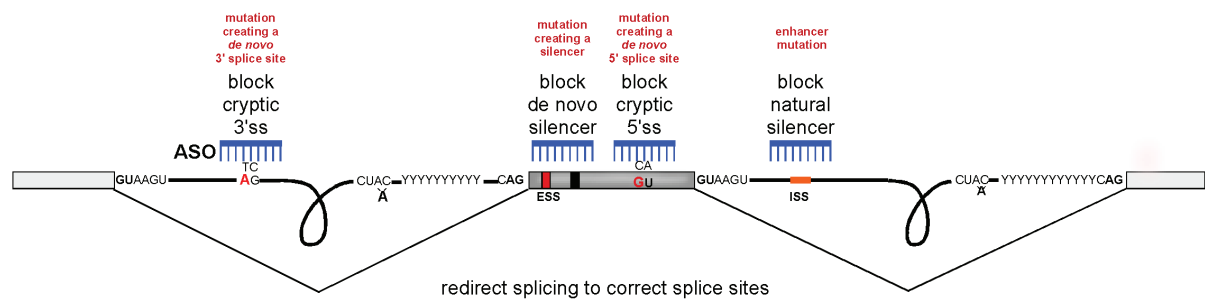
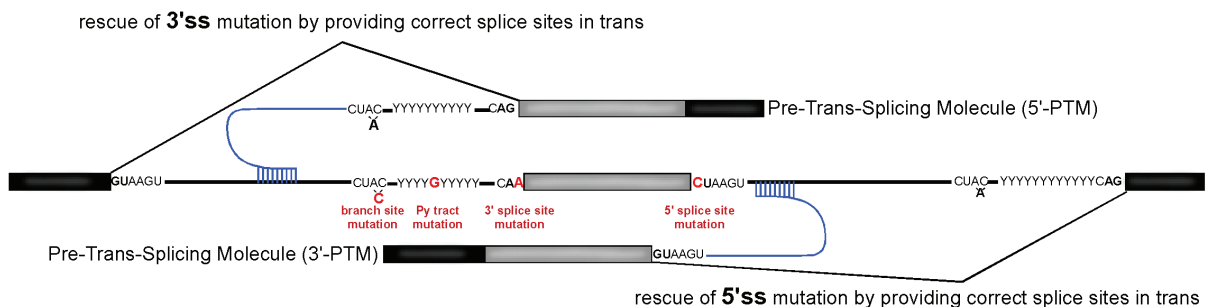
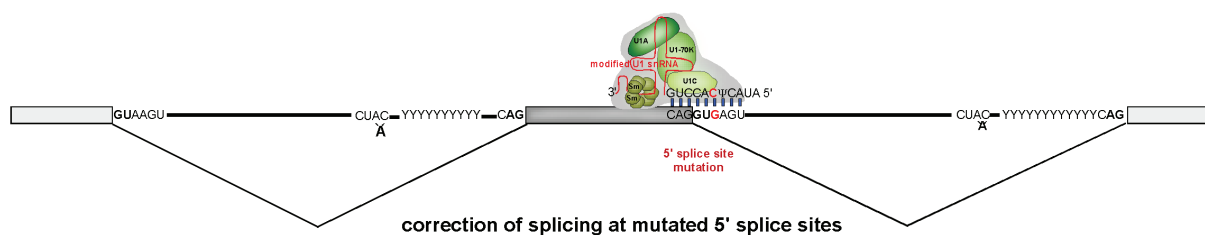
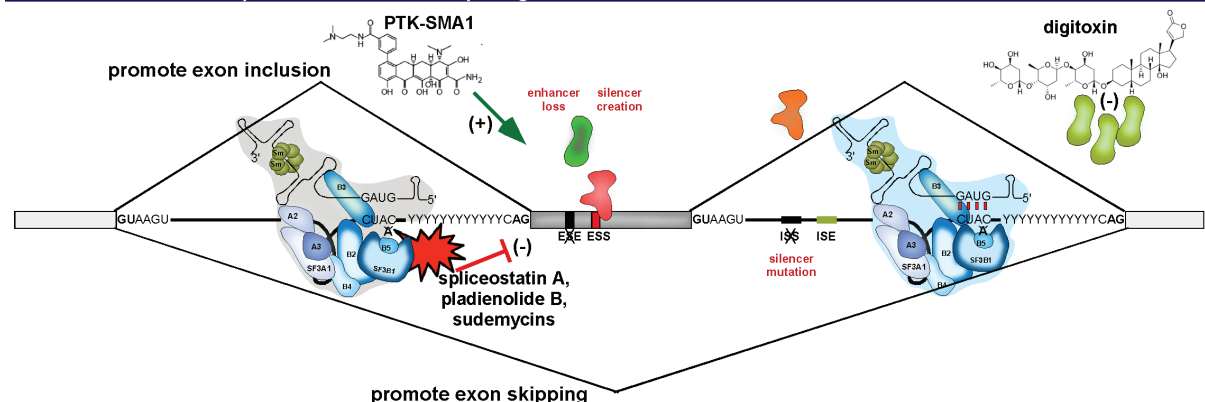
### Antisense Oligonucleotides (ASOs, AONs)

ASOs are short oligonucleotides, typically 15–25 bases in length, which are the reverse complement sequence of a specific RNA transcript target region. ASOs function by forming Watson–Crick base-pairs with the target RNA.<sup>10</sup> ASO binding to a target RNA sterically blocks access of splicing factors to the RNA sequence at the target site. Thus, an ASO targeted to a splice site will block splicing at the site, redirecting splicing to an adjacent site (Figure 2(a)). Alternatively, ASOs targeted to a splicing enhancer or silencer can prevent binding of *trans*-acting regulatory splicing factors at the target site and effectively block or promote splicing

(Figure 2(a)). ASOs have also been designed that can base-pair across the base of a splicing regulatory stem loop in order to strengthen the stem-loop structure.<sup>11</sup> The sequence specificity of ASOs allows them to bind precisely to endogenous RNAs and, importantly, their fidelity allows targeting of distinct RNA isoforms. ASOs have also been designed to target only mutated gene alleles, which will be particularly valuable in developing therapies for mutations causing autosomal dominant diseases. These features make ASOs a versatile tool that can be used to correct or alter RNA expression for therapeutic benefit.

In addition to their specificity, ASOs have many other features that make them an ideal therapeutic tool. For example, ASOs are relatively noninvasive therefore they do not alter the genome directly, and improvements in chemistries have been developed to improve the utility of ASOs as a therapeutic drug.<sup>10</sup> Current technologies utilize ASOs that are very stable, are efficiently and spontaneously internalized by cells *in vivo*, have high substrate specificity and low toxicity, and are not degraded by endogenous RNase H.<sup>10</sup> The half-life of naked ASOs in mouse and human plasma and many mouse tissues is approximately 10–15 days.<sup>10</sup> Remarkably, treatment of mice with a single injection of ASOs early in life has been shown to correct splicing and disease-associated phenotypes for up to a year.<sup>12,13</sup> The basis of this longevity is unclear. The enduring effect may be attributable to the stability of ASOs in post-mitotic cells where they are maintained and continue to influence splicing long after administration. Given the ease of delivery, favorable toxicity profile and enduring effects, ASOs are emerging as an ideal disease therapeutic.<sup>10</sup>

A number of variations on the basic ASO technology have been developed in order to tailor activity of the molecules after they base-pair to their target site. Such ASO modifications provide additional functions in cases where base-pairing is not sufficient for therapeutic splicing modification. A number of different dual-function or bifunctional ASOs have been designed. For example some hybrid ASOs have been developed in which the antisense base-pairing sequence is linked to a consensus binding site sequence for a specific splicing factor. In this way, the ASO can be directed to bind to the target RNA and also recruit a protein. This protein, depending on its function and the location where the ASO binds, can either enhance<sup>14</sup> or silence<sup>15,16</sup> splicing. ASOs have also been equipped with peptide sequences that provide an interaction domain to recruit splicing factors.<sup>17</sup> Recently, another type of bifunctional ASO was designed with a functional group, 2′deoxy-2′fluoro (2′F) nucleotide, that incidentally recruits the ILF2/3

**(a) ASO-based correction of mutation-induced aberrant splicing****(b) Trans-splicing rescue of mutation-induced aberrant splicing****(c) Modified U1 snRNA compensation of 5' splice site mutations****(d) Small molecule compounds that modulate splicing**

**FIGURE 2** | Tools to correct aberrant splicing caused by mutations. (a) ASO-based correction of mutation-induced aberrant splicing depicting ways in which ASOs (blue) can be used to correct for mutations (red) to promote proper splicing and exon inclusion. (b) *Trans*-splicing rescue of mutation-induced aberrant splicing depicting the replacement of the 3' or 5' portion of an RNA with mutated 3' or 5' splice sites, respectively, by a pre-trans-splicing molecule (PTM). Core splicing sequence mutations are depicted in red. (c) Modified U1 snRNA compensation for a 5' splice site mutation (red). Exogenous U1 snRNA with a compensatory mutation allows for base-pairing with the 5' splice site and the restoration of exon recognition and inclusion. (d) Small molecule compounds that modulate alternative splicing. Small molecules act in *trans* by binding spliceosome components to promote alternative exon inclusion to compensate for a mutation (examples noted in red lettering and represented with an X) or to alter mRNA isoforms for therapeutic benefit. Depicted are examples of small molecules that inhibit splicing (spliceostatin A, pladienolide B, and sudemycins) and two small molecules that promote exon inclusion (PTK-SMA1 and digitoxin).

protein, which has no known link to splicing. However, when targeted to bind near a 5'ss, the additional recruitment of ILF2/3 interfered with proper spliceosome assembly, generally resulting in the exclusion of the exon that borders the 5'ss from the mature mRNA.<sup>18</sup> In these examples, when proteins bind to an extended tail of an ASO or to the ASO backbone itself, the ASO acts as a portable splicing silencer or enhancer element (Figure 3(a)).

The ultimate goal in the development of many ASO-based therapeutics, traditional or bifunctional, is to either block the production of a toxic form of a protein or to restore the production of a protein that is not made or is nonfunctional. For this, ASOs are designed that base-pair to pre-mRNA and influence splicing in such a way as to alter the protein-coding mRNA (Figure 2(a)). ASOs can be used to restore a functional protein that was lost as a result of mutation. To restore function, ASOs can be used to block cryptic or pseudo splice sites or to promote the inclusion of an exon by targeting splicing regulatory sequences (Figure 2(a)). For example, to eliminate an mRNA isoform that produces a detrimental protein, or in cases where a mutation introduces a nonsense or frame-shift mutation, ASOs can be designed to promote usage of a cryptic splice site or to induce exon skipping to trigger the formation of a frame-shift and premature termination codon (PTC). Such aberrations typically elicit nonsense-mediated decay (NMD) and degradation of the entire mRNA, and hence disallow the production of the protein. An alternative approach is to bypass a mutation that introduces a nonsense or frame-shift mutation. ASOs can accomplish either of these alterations in splicing by base-pairing and blocking the interaction of the region with splicing factors that are required for splicing at a particular splice site (Figures 2 and 3).

### Trans-Splicing

*Trans*-splicing is currently under development as a therapeutic for a number of diseases.<sup>19</sup> The *trans*-splicing methodology, often referred to as spliceosomal-mediated RNA *trans*-splicing (SMaRT),<sup>19</sup> is designed to replace the entire coding sequence 5' or 3' of a target splice site. In this technology, a plasmid expresses a three-component pre-*trans*-splicing molecule, PTM. The PTM consists of an ASO that targets the endogenous intron of the mutated splice site, a synthetic splice site that directs splicing to bridge from the endogenous RNA to the PTM, and a copy RNA sequence that will be spliced to the endogenous RNA rather than to the mutated, inactive endogenous splice site. The ASO can be targeted either upstream or downstream of a consensus

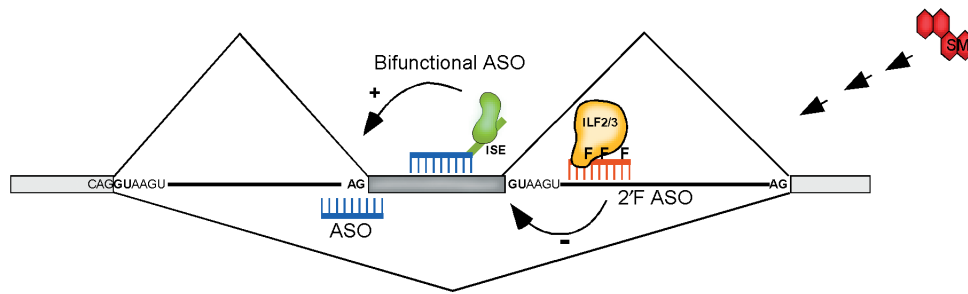
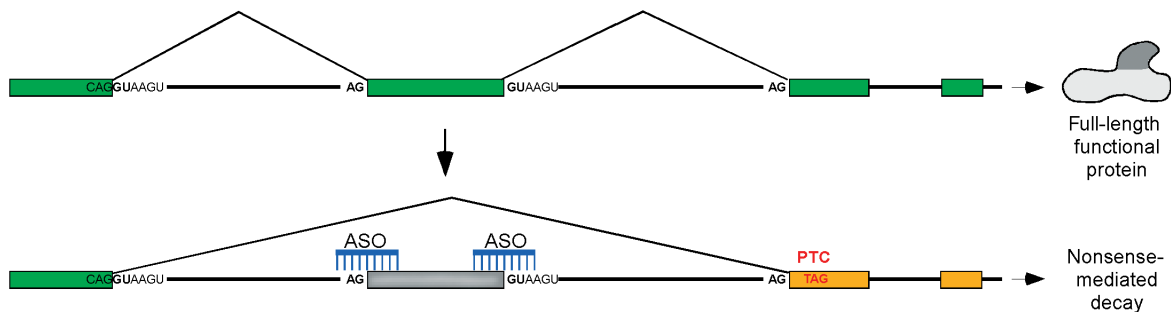
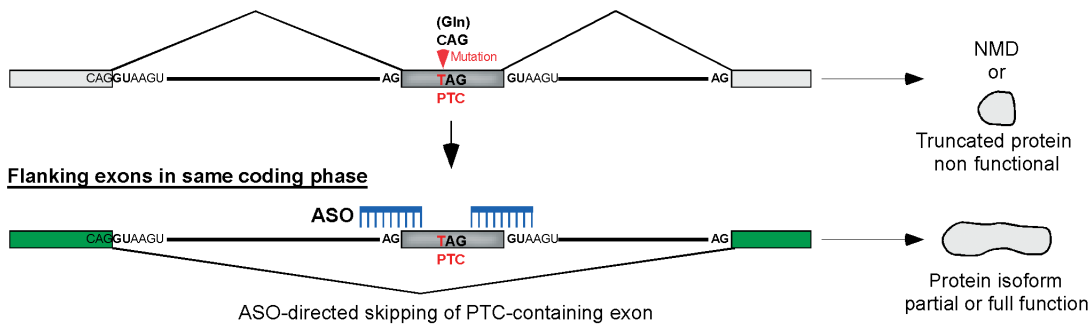
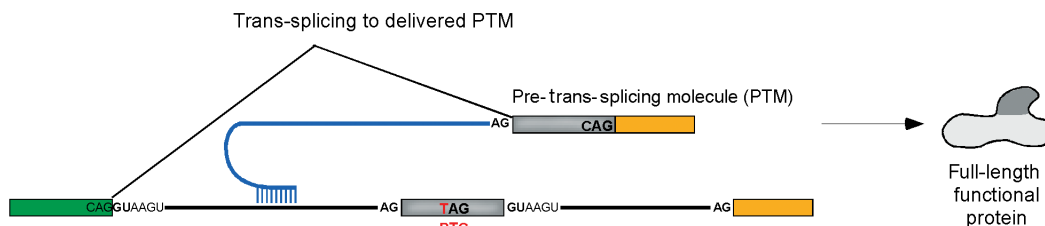
3'ss or 5'ss, respectively, depending on the location of the mutation, and the desired mRNA portion to be replaced (Figure 2(b)). PTMs are most often used to replace the 3' end of a mutated gene by utilizing the spliceosome to ligate the therapeutic coding sequence to the 5' sequence of the endogenous RNA. However, this method can also be used to replace an internal exon or the 5' region of a mutated gene<sup>19</sup> (Figure 2(b)).

*Trans*-splicing is an effective means to correct the expression of genes with mutations in the 5'ss or 3'ss that cannot be readily corrected using other technologies. In particular, *trans*-splicing is the best solution if a splice site mutation is in the first or last nucleotides of the intron. The base specificity at these positions is important for the catalytic steps of the splicing reaction, and thus, it is difficult to rescue splice site activity. For these types of mutations, the approach that has been effective at correcting gene expression is *trans*-splicing.

*Trans*-splicing approaches necessitate the delivery of DNA expression vectors to cells. Thus, complications in the delivery of PTMs are similar to those seen with the delivery of whole gene replacements.<sup>19</sup> One benefit to *trans*-splicing over gene replacement is that the expression of the pre-mRNA of the target gene remains under endogenous control. The PTM only replaces a portion of a gene and therefore the endogenous promoter controls the transcription of the pre-mRNA. The *trans*-splicing molecule can only interact with an existing pre-mRNA, and therefore the tissue-, temporal-, and quantity-specific expressions of the gene are not altered.<sup>19</sup> This technology is particularly useful when only small increases in therapeutic RNA are required, and some read-through of the mutated gene is tolerable. This is because 100% efficiency of *trans*-splicing may not be guaranteed, in part because of limitations in viral expression and delivery. PTMs can be especially beneficial in cases where an entire gene is too large to package and deliver to cells in a virus, but the gene's product can be corrected with a replacement of a portion of the RNA.

### Modified snRNAs

The U1 snRNA component of U1 snRNPs interacts with the 5'ss by specific base-pairing. A mutation in the 5'ss, which is a common disease-causing mutation, compromises U1 snRNA binding and can prevent spliceosome assembly and subsequent splicing (Figure 1(a)). To restore splicing, modified versions of the spliceosomal snRNAs have been created with sequence changes that restore base-pairing to the mutated 5'ss (Figure 2(c)). The complementary

**(a) Modulating alternative splicing****(b) Splicing redirection to knock-down gene expression****(c) Splicing-mediated rescue of gene expression disrupted by nonsense mutations****Flanking exons in different coding phases**

**FIGURE 3** | Tools used to induce aberrant splicing for disease therapy. (a) Modulating alternative splicing for disease therapy using ASOs, bifunctional ASOs (e.g., 2'F ASO), or small molecules (SM). These therapeutics can be used to either promote exon inclusion or skipping in the presence or absence of a mutation. (b) Targeting splicing to disrupt gene expression by the use of ASOs to promote exon skipping to disrupt the reading frame (green versus yellow exons) and promote nonsense-mediated decay (NMD). This process is known as forced-splicing-dependent nonsense-mediated decay (FSD-NMD). (c) Splicing-mediated rescue of gene expression disrupted by nonsense mutations. Top panel: The C to T point mutation that introduces a PTC either resulting in NMD, or a truncated, nonfunctional protein. Middle panel: The use of ASOs (blue) to block the 5' and 3' splice sites promotes exon skipping. The reading frame remains intact (green exons) resulting in a truncated but functional protein. Bottom panel: The use of a PTM to replace the 3' portion of the mRNA because the exons flanking the exon containing the PTC mutation are not in the same reading frame (green versus yellow exons). The use of the PTM results in a full-length and functional protein.

mutation allows the snRNA to effectively bind the mutant binding site of the pre-mRNA and restores normal splicing<sup>20</sup> (Figure 2(c)). A drawback of this approach is that the snRNAs must be incorporated into an expression vector and delivered to cells. Thus, this methodology has similar limitations as *trans*-splicing approaches.<sup>20</sup>

## Small Molecule Compounds

Small molecules can function by directly modifying the activity of splicing factors or by indirectly altering splicing, frequently by unknown mechanisms (Figure 2(d)). Small molecule effectors of specific splicing events are often identified using high-throughput screening assays using cells that report alterations in a particular splicing event.<sup>21</sup> However, frequently these molecules function in an indirect way on splicing. Thus, one drawback of small molecule therapeutics is the lack of specificity and information regarding the exact mechanism of action, which can lead to off-target effects. Nevertheless, a major benefit of some small molecules is that many are already approved and in use in clinical practice to treat diseases and conditions apart from splicing defects.<sup>22,23</sup> Therefore, these molecules have been deemed safe for use in humans, which vastly accelerates their development as a treatment for a disease. In fact, there are numerous classes of small molecules currently being studied for their ability to therapeutically alter splicing. Small molecules provide a valuable therapeutic tool to manipulate splicing, but lack specificity and can have undesirable off-target effects.

## TARGETING SPLICING IN DISEASE: THE FIXES

The tools described in the previous section have been used to manipulate splicing to achieve many different outcomes predicted to be therapeutically beneficial. Approaches for therapeutic RNA splicing interventions have focused either on correcting aberrant splicing that is associated with disease, or on inducing aberrant splicing in a way that can ameliorate disease phenotypes.

## Correction of Aberrant Splicing

### Splice Site Mutations

Mutations within the consensus sequence of the 5'ss, 3'ss, or the BPS that inactivate splicing are common. The approaches used to correct these mutations require careful consideration of the location of the

mutation and its effect on splicing. Mutations in consensus splice site sequences are often catastrophic for splicing because these nucleotides, in particular the first and last dinucleotides of the intron, function specifically in the catalytic steps of splicing. Several different approaches are being used to correct the defects associated with splice site mutations (Figure 2).

### Trans-Splicing

*Trans*-splicing is the only RNA-targeting approach that has been effectively employed to rescue full-length gene expression that has been disrupted by mutations in the terminal dinucleotides of the intron. In *trans*-splicing approaches, the PTM molecule can be designed to deliver and express *in trans* the 5' or 3' half of the RNA transcript flanking the mutated sequence. This PTM molecule includes a sequence complementary to the intronic RNA for targeting and a consensus splice site which splices the PTM to the endogenous transcript, effectively producing a full-length, functional mRNA (Figure 2(b)). In *trans*-splicing, the specific mutation is not relevant because the PTM delivers the wild-type sequence. This approach has been used to replace the first exon of the *HBB* ( $\beta$ -globin) gene as a therapy for  $\beta$ -thalassemia<sup>24</sup> (Table 1, splice site mutations).

### Modified snRNAs

Modified U1 snRNAs have been used to restore splice site recognition<sup>34</sup> of the mutated and inactive U1 snRNA binding site in the 5'ss (Figure 2(c)). In this case, a modified U1 snRNA, which carries the compensatory mutation to the mutation at the pre-mRNA splice site, is introduced to cells. Modified U1 snRNAs have been used successfully to rescue splicing at mutated 5'ss in diseases including cystic fibrosis,<sup>27</sup> hemophilia B,<sup>27</sup> F7 deficiency,<sup>30</sup> Fanconi anemia,<sup>34</sup> Bardet-Biedl syndrome,<sup>25</sup> propionic acidemia,<sup>35</sup> and retinitis pigmentosa<sup>36,37</sup> (Table 1, splice site mutations). The restoration of these splicing events allows for the production of functional proteins and has potential to improve disease phenotype. However, to date, modified snRNAs have not successfully corrected splicing at a 5'ss mutated at the first or second nucleotides of the intron, likely because of the requirement of these specific nucleotides in splicing catalysis for function in addition to snRNA binding.

### ASOs

ASOs have been used to restore gene expression in the context of a core splice site mutation. For example, ASOs provide a viable therapeutic approach in cases where a disabling splice site mutation activates a cryptic splice site or triggers exon skipping that

**TABLE 1** | Examples of Splicing-Based Therapeutic Approaches

Disease	Human Target Gene	Therapeutic	Stage	References
Correction of Aberrant Splicing				
<i>Splice Site Mutations</i>				
Bardet–Biedl syndrome	<i>BBS1</i>	U1/U6 snRNA <sup>1</sup>	Patient cells	20,25
$\beta$ -Thalassemia	<i>HBB</i>	PTM	Minigene	24
Cancer	<i>BRCA1</i>	ASO	Minigene	26
	<i>PTCH1</i>	ASO	Minigene	26
Cystic fibrosis	<i>CFTR</i>	U1 snRNA <sup>1</sup>	Minigene	27
Duchenne muscular dystrophy	<i>DMD</i>	ASO	Canine model	28,29
Factor VII deficiency	<i>F7</i>	U1 snRNA <sup>1</sup>	Minigene	30
Familial dysautonomia	<i>IKBKAP</i>	SM	Patients	31–33
Fanconi anemia	<i>FANCC</i>	U1 snRNA <sup>1</sup>	Patient cells	34
Hemophilia A	<i>F9</i>	U1 snRNA <sup>1</sup>	Minigene	27
Propionic acidemia	<i>PCCA</i>	U1 snRNA <sup>1</sup>	Patient cells	35
Retinitis pigmentosa	<i>RHO</i>	U1 snRNA <sup>1</sup>	Minigenes	36
	<i>RPGR</i>	U1 snRNA <sup>1</sup>	Patient cells	37
<i>Cryptic Splice Sites</i>				
Ataxia telangiectasia	<i>ATM</i>	ASO	Patient cells	38
$\beta$ -Thalassemia	<i>HBB</i>	ASO	Mouse model	39–44
Cancer	<i>BRCA2</i>	ASO	Minigene	45
CDG1A <sup>2</sup>	<i>PMM2</i>	ASO	Patient cells	46
Congenital adrenal insufficiency	<i>CYP11A</i>	ASO	Minigene	26
Cystic fibrosis	<i>CFTR</i>	ASO	Cell lines	47
		SM	Patient cells	48
Duchenne muscular dystrophy	<i>DMD</i>	ASO	Patient cells	49
Fukuyama congenital muscular dystrophy (FCMD)	<i>FKTN</i>	ASO	Mouse model	50
Growth hormone insensitivity	<i>GHR</i>	ASO	Minigene	51
HPABH4A <sup>2</sup>	<i>PTS</i>	ASO	Patient cells	52
Hutchinson–Gilford progeria (HGPS)	<i>LMNA</i>	ASO	Mouse model	53,54
MLC1 <sup>2</sup>	<i>MLC1</i>	ASO	Minigene	55
Methylmalonic aciduria	<i>MUT</i>	ASO	Patient cells	56,57
Myopathy with lactic acidosis	<i>ISCU</i>	ASO	Patient cells	58,59
Myotonic dystrophy	<i>CLC1</i>	ASO	Mouse model	60
Neurofibromatosis	<i>NF1</i>	ASO	Patient cells	61
Niemann–Pick type C	<i>NPC1</i>	ASO	Patient cells	62
Propionic acidemia	<i>PCCB</i>	ASO	Patient cells	57
Usher syndrome	<i>USH1C</i>	ASO	Mouse model	63
<i>Regulatory Sequence Mutations</i>				
Afibrinogenemia	<i>FGB</i>	ASO	Minigene	64
Cancer	<i>BRCA1</i>	ASO	In vitro	17
Propionic acidemia	<i>PCCA</i>	ASO	Patient cells	57
Neurofibromatosis	<i>NF1</i>	SM	Patient cells	65
Ocular albinism type 1	<i>GRP143</i>	ASO	Patient cells	66

TABLE 1 | Continued

Disease	Human Target Gene	Therapeutic	Stage	References
<i>Deregulated Alternative Splicing</i>				
Alzheimer's disease/FTDP-17 Tauopathies	<i>MAPT</i>	ASO	Cell lines	11,18,67
		PTM	Minigene	68
		SM	Cell lines	22
Cancer	<i>BCL2L1</i>	ASO	Mouse model	18,69–72
	<i>FGFR1</i>	ASO	Cell lines	73
	<i>MCL1</i>	ASO	Cell lines	74
	<i>MDM2</i>	ASO	Cell lines	75
	Multiple	SM	Cell lines	76,77
	<i>PKM</i>	ASO	Cell lines	18,78
	<i>MST1R</i>	ASO	Cell lines	79
	<i>USP5</i>	ASO	Cell lines	80
Spinal muscular atrophy	<i>SMN2</i>	ASO	Clinical trials phase Ib/IIa, ISIS-SMNRx	12,13,17,18, 81–83
		SM	Clinical trials	84,85
		U1 snRNA <sup>1</sup>	Minigene	27
		PTM	Mouse model	86,87
<i>Induction of Aberrant Splicing</i>				
<i>Knockdown of Detrimental Gene Expression</i>				
Alzheimer's disease	<i>BACE1</i>	ASO	Cell lines	88
Cancer	<i>CDKN1A</i>	SM	Cell lines	89
	<i>ERBB2</i>	ASO	Cell lines	90,91
	<i>FLT1</i>	ASO	Mouse model	92
	<i>HNRNPH1</i>	ASO	Patient cells	93
	<i>KDR</i>	ASO	Mouse model	94,95
	<i>MYC</i>	SM	Cell lines	96
	Multiple	SM	Clinical trials phase I, E7107	97–105
	<i>PHB</i>	SM	Cell lines	103
	<i>SRA1</i>	ASO	Cell lines	106
	<i>STAT3</i>	ASO	Mouse model	107
	<i>TERT</i>	ASO	Cell lines	108
	<i>WT1</i>	ASO	Cell lines	109
FHBL/atherosclerosis <sup>2</sup>	<i>APOB</i>	ASO	Cell lines	110
Immune-response	<i>CD40</i>	ASO	Cell lines	111
Inflammatory disease	<i>TNFRSF1B</i>	ASO	Mouse model	112
	<i>IL5RA</i>	ASO	Cell lines	113
Influenza virus	<i>TMPRSS2</i>	ASO	Cell lines	114
Muscle wasting diseases	<i>MSTN</i>	ASO	Mouse model	115
Spinocerebellar ataxia type 1	<i>ATXN1</i>	ASO	Cell lines	116

**TABLE 1** | Continued

Disease	Human Target Gene	Therapeutic	Stage	References
<i>RNA Reframing</i>				
Duchenne muscular dystrophy	<i>DMD</i>	ASO	Clinical trials phase II, PRO051, AVI-4658, PRO044	49,117–126
Dystrophic epidermolysis bullosa	<i>COL7A1</i>	SM	Cell lines	127
		ASO	Explants	128
		PTM	Patient cells	129
Miyoshi myopathy	<i>DYSF</i>	ASO	Patient cells	130

SM, small molecule; PTM, pre-*trans*-splicing molecule; ASO, antisense oligonucleotide.

HUGO gene names are provided. The terms under Stage refers to the systems used in the most advanced studies for a particular gene or disease. Patient cells refer to studies performed using cells isolated from patients with the disease mutation; minigene refers to studies using a cloned portion of the disease gene; cell lines indicate conventional, transformed, high-passage number cell lines; explants refer to mouse tissue.

<sup>1</sup>Modified.

<sup>2</sup>Full disease names are as follows: CDG1A, congenital disorder of glycosylation, type Ia; HPABH4A, hyperphenylalaninemia, BH4-deficient, A; MLC1, megalencephalic leukoencephalopathy with subcortical cysts; FHBL, Hypobetalipoproteinemia.

produces an mRNA with a frame-shift. In this situation, an ASO can be designed to redirect splicing to reframe the transcript and produce an mRNA with a restored reading frame that codes for a protein isoform with partial or full function. This approach has been used successfully to correct gene expression disrupted by a 3'ss mutation that causes exon skipping and consequently introduces a PTC in the *DMD* gene that causes Duchenne muscular dystrophy<sup>28,29</sup> (Table 1, splice site mutations).

Splice site mutations can also result in the activation of a pseudo splice site that is recognized by the spliceosome preferentially over the mutated site. In these cases, ASOs can be targeted to hybridize to the pseudo splice site and sterically block its activity. If there are no other cryptic splice sites that are stronger than the mutated authentic splice site, the ASO block may reactivate the mutated authentic splice site. This technique has been used successfully for 5'ss mutations in *BRCA1* and *PTCH1*.<sup>26</sup> These types of splice site mutations have also been rescued using small molecules that can promote splicing to the inactivated splice site. An example of such a molecule is kinetin, which can partially rescue splicing to a mutated *IKBKAP* 5'ss in familial dysautonomia<sup>31,32</sup> (Table 1, mutated splice sites).

### Cryptic/Pseudo Splice Sites

The creation of *de novo* cryptic splice sites and the activation of pseudo splice sites are common consequences of disease-causing mutations. A cryptic splice site is the one created by a mutation, whereas a pseudo site is a site with a weak match to consensus splice site which is activated as a result of a mutation in the authentic site or in regulatory

splicing sequences.<sup>8,131</sup> The consequences of these types of mutations as well as the potential approaches to compensate for the mutations are similar, and thus the terms should be considered interchangeable as we discuss the approaches to target them (Figure 1).

Cryptic or pseudo splice sites result in the inclusion of intronic sequences typically not included in the mature mRNA (pseudoexons). The resulting aberrant mRNA may have an altered reading frame or may code for a protein with amino acid deletions or additions. Frame-shifts can result in PTCs and/or a complete alteration of the amino acid sequence encoded in the mRNA. This is a well-documented phenomenon, and mutations in numerous human genes lead to the use of cryptic splice sites and the inclusion of pseudoexons, resulting in disease.<sup>131</sup>

### ASOs

ASOs are the most common therapeutic approach to correct splicing alterations caused by the creation of a cryptic splice site. ASOs can be designed to bind and thereby occupy the cryptic splice site, which redirects the spliceosome to the authentic splice site. Blocking cryptic 5'ss with ASOs has been used in several disease models to correct splicing (Table 1, cryptic splice sites). ASO-directed blocking of cryptic splice sites caused by mutations in *HBB* ( $\beta$ -globin), *FKTN*, *LMNA*, and *CLC1* has been shown to rescue normal splicing in mouse models of  $\beta$ -thalassemia,<sup>39–43</sup> Fukuyama congenital muscular dystrophy,<sup>50</sup> Hutchinson-Gilford progeria,<sup>53</sup> and myotonic dystrophy,<sup>60</sup> respectively (Table 1, cryptic splice sites). Also, rescue of disease phenotype in a mouse model has been shown using ASOs targeting a mutation the *USH1C* gene that creates a cryptic

5'ss and causes Usher syndrome. These ASOs have successfully corrected splicing and rescued congenital deafness and vestibular defects associated with the disease *in vivo*.<sup>63</sup>

### Small Molecules

Small molecule compounds have also been identified that restore authentic splice site recognition or prevent the recognition of pseudoexons. For example, the histone deacetylase inhibitor, sodium butyrate, promotes exon inclusion in *CFTR*, that causes cystic fibrosis, in cell lines carrying the 3849+10kbC>T mutation and restores functional *CFTR* channels<sup>48</sup> (Table 1, cryptic splice sites). The molecule appears to act by increasing expression of a subset of splicing factors.

### Splicing Regulatory Sequence Mutations

Splicing mutations that disrupt *cis*-acting regulatory sequences such as splicing enhancers and silencers can be difficult to identify and are often designated as missense mutations, or as single nucleotide polymorphisms (SNPs), if located within exons. When these sequence elements are mutated, the recognition of authentic splice sites is affected, resulting in non-canonical exon skipping or pseudo splice site recognition (Figure 1(b)). Alternatively, *de novo cis*-acting regulatory sequences can also be generated by mutations. Once a mutation is determined to alter splicing regulatory sequence, a number of approaches can be considered to correct the splicing defect. Approaches can include blocking activated splice sites to balance the loss of the enhancer by identifying and targeting splicing silencers, or by restoring the enhancer or silencer regulation at the mutated site (Figure 2(a)).

### ASOs

ASOs have been the primary tool used to correct splicing defects associated with the loss or creation of regulatory sequences such as splicing silencers and enhancers (Table 1, regulatory sequence mutations). For example, mutations that create binding sites for the splicing factor SRSF1 in the *FGB* or the *GPR143* (*OA1*) genes cause cryptic splicing that causes afibrinogenemia<sup>64</sup> or ocular albinism,<sup>66</sup> respectively. These cryptic sites have been blocked through direct ASO hybridization and to redirect splicing to the authentic splice site. Another SRSF1 site disrupted by a mutation in *BRCA1* causes exon skipping, which promotes breast cancer. Bifunctional ASOs that replace the need for SRSF1 binding have also been designed to include an RS peptide sequence that mimics a functional RS domain of SRSF1, and in

so doing, effectively restores exon recognition in the mutated *BRCA1* gene.<sup>17</sup>

### Small Molecules

Small molecule compounds often function through interactions with splicing factors. Thus, they are also a viable option for the treatment of diseases caused by mutations that affect *cis*-acting regulatory sequences (Figure 2(d)). The small molecule kinetin, for example, can restore splicing of several exons in the *NF1* gene that are skipped in neurofibromatosis as a result of mutations in *cis*-acting sequences<sup>65</sup> (Table 1, regulatory sequence mutations).

### Deregulated Alternative Splicing

For the purposes of this review, disease-associated disruption of alternative splicing refers to any pathological alteration in the relative abundance of alternatively spliced mRNA isoforms. In these cases, the splicing defect is the one that disrupts the balance of normally spliced transcripts as opposed to examples discussed earlier which consider mutations that result in the creation of *de novo*-spliced mRNA that is not typically observed. Changes in alternative splicing can be caused by mutations within the sequence of the alternatively spliced gene transcript or in the absence of mutations within the genes. In the latter situation, it is often difficult to assign an exact cause of the change in alternative splicing. Possibilities include the malfunction of a signaling pathway or disruption of some other indirect regulatory event.

### ASOs, Trans-Splicing and Small Molecules

The utilization of RNA splicing-based approaches to alter spliced isoforms has been particularly successful in the development of therapeutics for spinal muscular atrophy (SMA). SMA patients have lost expression of the survival of motor neuron (SMN) protein transcripts from the *SMN1* gene. An *SMN1* paralog, *SMN2*, expresses SMN encoding transcripts. However, a silent mutation in exon 7 of the gene results in the preferential skipping of exon 7 thus generating a truncated and unstable protein. Promoting *SMN2* exon 7 inclusion to restore full protein function has been a major focus of SMA therapeutic development.<sup>84</sup> Most of the tools discussed in this review have been used to manipulate exon 7 splicing in SMA, including ASOs, small molecule compounds and *trans*-splicing.<sup>84</sup> ASOs targeting different regions surrounding exon 7, both in the exon and flanking intron, have been developed.<sup>12,81,82,132–134</sup> One ASO, ISIS-SMNrx, is now in clinical trials.<sup>82</sup>

Many small molecule compounds have also been identified that increase the amount of SMN

protein from the *SMN2* gene in SMA<sup>84</sup> (Table 1, deregulated alternative splicing). At least one of these compounds, the tetracycline derivative, PTK-SMA1,<sup>135</sup> acts directly on the splicing reaction through an unknown target, most of these compounds alter splicing indirectly via changes in transcriptional activity or other unknown mechanisms as reviewed by Bebee et al.<sup>84,85</sup> (Table 1). Some of these molecules have progressed to clinical trials.<sup>84</sup>

Modulating the production of alternatively spliced isoforms for disease therapy has shown promise in other diseases including Alzheimer's disease (AD) and numerous cancers (Table 1, deregulated alternative splicing). An increase in *MAPT* exon 10 splicing is associated with AD and FTDP-17. A number of approaches are aimed at promoting exon 10 skipping to increase the production of the shorter isoform. Platforms include traditional and bifunctional ASOs,<sup>11,18,67</sup> *trans*-splicing,<sup>68</sup> and small molecules such as the cardionic steroid, digitoxin, that promotes exon 10 skipping by altering the expression of key splicing factors<sup>22,136</sup> (Figure 2). Likewise, a number of deregulated alternative splicing events associated with tumor progression have been the focus of ASO-based splicing redirection to promote splicing of anti-tumorigenic isoforms of genes such as *BCL2L1* (*BCL-X*),<sup>18,69–72</sup> *FGFR1*,<sup>73</sup> *MCL-1*,<sup>74</sup> *MDM2*,<sup>75</sup> *PKM*,<sup>18,78</sup> *MST1R* (*RON*),<sup>79</sup> and *USP5*<sup>80</sup> (Figure 3(a)). In cancer cells, where global aberrant alternative splicing is thought to contribute to the oncogenic potential, small molecule compounds have also been identified that promote normalization of alternative splicing events as a potential therapy.<sup>76,77</sup>

## Induction of Aberrant Splicing

RNA processing can be targeted not only to correct the effects of mutations or deregulated splicing, as discussed above, but also to induce splicing events that do not normally occur. This approach has proven to be an effective and powerful way to deliberately alter or eliminate protein expression in ways that can be therapeutically valuable (Figure 3(b) and (c)).

### Detrimental Gene Expression

In some diseases, therapeutic benefit can be achieved by eliminating an mRNA isoform and the encoded protein altogether. In this case, the goal of inducing aberrant splicing is to cause transcript degradation and/or protein truncation yielding no protein or a nonfunctional protein, respectively. The exclusion of specific exons from a transcript can cause a frameshift and introduce a PTC into the transcript if the exons flanking the skipped exon are not in the same

reading frame. An induced PTC is expected to induce the NMD pathway and result in the degradation of the transcript thus preventing protein production (Figure 3(b)).

### ASOs

ASOs have been used effectively to achieve this so-called forced splicing-induced nonsense-mediated decay (FSD-NMD).<sup>93,94,107</sup> This approach has been used to down-regulate *STAT3*,<sup>107</sup> *HNRNPH1*,<sup>93</sup> and *KDR*<sup>94,95</sup> to mitigate tumor progression, malignancy, and angiogenesis. FSD-NMD has also been used in muscle wasting disease and spinocerebellar ataxia type 1 to cause degradation of *MSTN*<sup>115</sup> and *ATXN1*,<sup>116</sup> respectively (Table 1, detrimental gene expression).

To determine whether knockdown of gene expression using FSD-NMD or splicing redirection will have the greatest therapeutic potential, the two processes can be compared. For example, by comparing these two approaches for *STAT3*,<sup>107</sup> and *KDR*,<sup>94</sup> mentioned above, splicing redirection was more effective at blocking cell proliferation than the complete knockdown. The greater effectiveness was achieved owing to the dual effects of eliminating a pathogenic RNA isoform and increasing a beneficial RNA isoform.

In some cases redirection of splicing to an aberrant isoform that is not normally generated from a gene transcript can be an effective means to eliminate a detrimental RNA isoform that is implicated in disease (Table 1, detrimental gene expression). Some of the more advanced studies, showing promise in animal models, include ASOs targeting *TNFRSF1B*<sup>112</sup> for the treatment of inflammatory disease, and *FLT1*<sup>92</sup> as a cancer therapy.

### Small Molecules

Induction of aberrant splicing for disease benefit has also been achieved using small molecules. Large-scale compound screening has identified many small molecules that regulate splicing, *per se*, rather than individual genes (Figure 3(a)). These molecules have been applied to the treatment of cancers. In general, these compounds are nonspecific, altering the splicing of multiple genes. For example, the small molecules, spliceostatin A (SSA; a derivative of FR901464) and pladienolide B (E7107) bind the SF3B spliceosomal complex, an essential component of the U2 snRNP<sup>97,98</sup> (Figure, 2(d), Table 1, detrimental gene expression). These small molecules and the closely related derivatives, meayamycin and herboxidiene (GEX1A), act as general inhibitors of splicing.<sup>97–99,137</sup> However, SSA as well as analogs of

SSA, sudemycins, have also been shown to modulate alternative splicing.<sup>100,101</sup> One of these inhibitors, E7107, has entered phase I clinical trials.<sup>98</sup> However, the value of SF3B inhibitors as a therapeutic is not clear because haploinsufficiency of SF3B1, a component of the SF3B complex, causes cancer,<sup>138</sup> and at least one chemical analog of spliceostatin A, meayamycin, induces abnormalities in normal cells.<sup>102</sup> Other molecules, such as histone deacetylase inhibitors<sup>103</sup> and C6 pyridinium ceramide,<sup>104</sup> can also induce changes in alternative splicing by altering the expression or activity of specific splicing factors (Table 1). All of these small molecules serve to alter alternative splicing and therefore disrupt the metabolic and proliferative activities of cancerous cells (Table 1, detrimental gene expression).

### RNA Reframing

Nonsense and small deletions/insertions can create PTCs or cause frame-shifts, both of which lead to the production of a truncated protein or the loss of gene expression as a result of NMD. In cases where the flanking introns are in phase, skipping the exon with the PTC or frame-shift mutation will produce an mRNA encoding an in-frame or reframed protein that is partially or fully functional (Figure 3(c)). Rescuing gene expression in this way has been accomplished using a number of tools including ASOs targeted to the splice sites, small molecule effectors of exon skipping and *trans*-splicing. Promising results from these frame-correcting approaches have been achieved for dystrophic epidermolysis bullosa,<sup>128,129</sup> and Miyoshi myopathy<sup>130</sup> (Table 1, reframing RNA).

One of the most actively pursued ASO-based therapeutics has been used for the treatment

of Duchenne muscular dystrophy (DMD). Nonsense mutations are common in DMD and ASOs designed to skip a variety of exons are being developed.<sup>49,117–125</sup> The most advanced ASO-based therapies to treat DMD, PRO051 (GSK 2402968),<sup>121</sup> AVI-4658 (Eteplirsen),<sup>117,119</sup> and PRO044<sup>122,126</sup> are currently in phase II clinical trials. PRO051 and AVI-4648 both induce the skipping of exon 51, which restores the reading frame of dystrophin for patients with deletions of either exon(s) 50 or 52; or deletions that span exons 45–50, or 48–50, which represents a patient population that accounts for 13% of DMD cases.<sup>117,121</sup> Similarly, PRO044 skips exon 44, which can restore the dystrophin reading frame in patients with deletions of either exons 43, 45, or deletions spanning exons 38–43, 40–43, 42–43, or 45–54, which account for 6% of DMD cases.<sup>126</sup>

### CONCLUSION

This review provides an overview of the different types of aberrant splicing that can occur as a result of mutations or other cellular defects, and highlights approaches that can be used to target and correct deficiencies by manipulating RNA splicing. Approaches that induce aberrant splicing for disease therapy are also considered. We have made an effort to be thorough, but acknowledge that this account is by no means comprehensive and we regret the omission of other important work that is not included because of space limitations. Given the success of RNA-based approaches, there will likely be an increasing number of diseases that will benefit from splice-altering therapeutics.

### ACKNOWLEDGMENTS

We thank Dr. Alicia Case and Anthony Hinrich for their critical reading of the manuscript. This work was supported by the Hearing Health Foundation, Midwest Eye-Banks, Capita Foundation, National Organization for Hearing Research Foundation and NIH NS069759 to MLH, NIH 1F31NS076237 to MAH, and the Schweppe Research Foundation and American Cancer Society, Illinois Division, Research Grant 189903 to DMD.

### REFERENCES

1. De Conti L, Baralle M, Buratti E. Exon and intron definition in pre-mRNA splicing. *WIREs: RNA* 2012, 4:49–60.
2. Wahl MC, Will CL, Luhrmann R. The spliceosome: design principles of a dynamic RNP machine. *Cell* 2009, 136:701–718.
3. Nilsen TW, Graveley BR. Expansion of the eukaryotic proteome by alternative splicing. *Nature* 2010, 463:457–463.
4. Kim E, Goren A, Ast G. Alternative splicing: current perspectives. *Bioessays* 2008, 30:38–47.
5. Kelemen O, Convertini P, Zhang Z, Wen Y, Shen M, Falaleeva M, Stamm S. Function of alternative splicing. *Gene* 2012, 514:1–30.

6. Lim KH, Ferraris L, Filloux ME, Raphael BJ, Fairbrother WG. Using positional distribution to identify splicing elements and predict pre-mRNA processing defects in human genes. *Proc Natl Acad Sci U S A* 2011, 108:11093–11098.
7. Sterne-Weiler T, Howard J, Mort M, Cooper DN, Sanford JR. Loss of exon identity is a common mechanism of human inherited disease. *Genome Res* 2011, 21:1563–1571.
8. Roca X, Sachidanandam R, Krainer AR. Intrinsic differences between authentic and cryptic 5' splice sites. *Nucleic Acids Res* 2003, 31:6321–6333.
9. Stenson PD, Mort M, Ball EV, Howells K, Phillips AD, Thomas NS, Cooper DN. The Human Gene Mutation Database: 2008 update. *Genome Med* 2009, 1:13.
10. Bennett CF, Swayze EE. RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform. *Annu Rev Pharmacol Toxicol* 2010, 50:259–293.
11. Peacey E, Rodriguez L, Liu Y, Wolfe MS. Targeting a pre-mRNA structure with bipartite antisense molecules modulates tau alternative splicing. *Nucleic Acids Res* 2012, 40:9836–9849.
12. Porensky PN, Mitrapant C, McGovern VL, Bevan AK, Foust KD, Kaspar BK, Wilton SD, Burghes AH. A single administration of morpholino antisense oligomer rescues spinal muscular atrophy in mouse. *Hum Mol Genet* 2012, 21:1625–1638.
13. Hua Y, Sahashi K, Rigo F, Hung G, Horev G, Bennett CF, Krainer AR. Peripheral SMN restoration is essential for long-term rescue of a severe spinal muscular atrophy mouse model. *Nature* 2011, 478:123–126.
14. Skordis LA, Dunkley MG, Yue B, Eperon IC, Muntori F. Bifunctional antisense oligonucleotides provide a trans-acting splicing enhancer that stimulates SMN2 gene expression in patient fibroblasts. *Proc Natl Acad Sci U S A* 2003, 100:4114–4119.
15. Osman EY, Yen PF, Lorson CL. Bifunctional RNAs targeting the intronic splicing silencer N1 increase SMN levels and reduce disease severity in an animal model of spinal muscular atrophy. *Mol Ther* 2012, 20:119–126.
16. Meyer K, Marquis J, Trub J, Nlend R, Nlend R, Verp S, Ruepp MD, Imboden H, Barde I, Trono D, Schumperli D. Rescue of a severe mouse model for spinal muscular atrophy by U7 snRNA-mediated splicing modulation. *Hum Mol Genet* 2009, 18:546–555.
17. Cartegni L, Krainer AR. Correction of disease-associated exon skipping by synthetic exon-specific activators. *Nat Struct Biol* 2003, 10:120–125.
18. Rigo F, Hua Y, Chun SJ, Prakash TP, Krainer AR, Bennett CF. Synthetic oligonucleotides recruit ILF2/3 to RNA transcripts to modulate splicing. *Nat Chem Biol* 2012, 8:555–561.
19. Wally V, Murauer EM, Bauer JW. Spliceosome-mediated trans-splicing: the therapeutic cut and paste. *J Invest Dermatol* 2012, 132:1959–1966.
20. Schmid F, Hiller T, Korner G, Glaus E, Berger W, Neidhardt J. A gene therapeutic approach to correct splice defects with modified U1 and U6 snRNPs. *Hum Gene Ther* 2012, 24:97–104.
21. Arslan AD, He X, Wang M, Rumschlag-Booms E, Rong L, Beck WT. A high-throughput assay to identify small-molecule modulators of alternative pre-mRNA splicing. *J Biomol Screen* 2012, 18:180–190.
22. Stoilov P, Lin CH, Damoiseaux R, Nikolic J, Black DL. A high-throughput screening strategy identifies cardiotonic steroids as alternative splicing modulators. *Proc Natl Acad Sci U S A* 2008, 105:11218–11223.
23. Warf MB, Nakamori M, Matthys CM, Thornton CA, Berglund JA. Pentamidine reverses the splicing defects associated with myotonic dystrophy. *Proc Natl Acad Sci U S A* 2009, 106:18551–18556.
24. Kierlin-Duncan MN, Sullenger BA. Using 5'-PTMs to repair mutant  $\beta$ -globin transcripts. *RNA* 2007, 13:1317–1327.
25. Schmid F, Glaus E, Barthelmes D, Fliegauf M, Gaspar H, Nurnberg G, Nurnberg P, Omran H, Berger W, Neidhardt J. U1 snRNA-mediated gene therapeutic correction of splice defects caused by an exceptionally mild BBS mutation. *Hum Mutat* 2011, 32:815–824.
26. Uchikawa H, Fujii K, Kohno Y, Katsumata N, Nagao K, Yamada M, Miyashita T. U7 snRNA-mediated correction of aberrant splicing caused by activation of cryptic splice sites. *J Hum Genet* 2007, 52:891–897.
27. Fernandez Alanis E, Pinotti M, Dal Mas A, Balestra D, Cavallari N, Rogalska ME, Bernardi F, Pagani F. An exon-specific U1 small nuclear RNA (snRNA) strategy to correct splicing defects. *Hum Mol Genet* 2012, 21:2389–2398.
28. Yokota T, Hoffman E, Takeda S. Antisense oligo-mediated multiple exon skipping in a dog model of duchenne muscular dystrophy. *Methods Mol Biol* 2011, 709:299–312.
29. Bish LT, Sleeper MM, Forbes SC, Wang B, Reynolds C, Singletary GE, Trafny D, Morine KJ, Sanmiguel J, Cecchini S, et al. Long-term restoration of cardiac dystrophin expression in golden retriever muscular dystrophy following rAAV6-mediated exon skipping. *Mol Ther* 2012, 20:580–589.
30. Pinotti M, Rizzotto L, Balestra D, Lewandowska MA, Cavallari N, Marchetti G, Bernardi F, Pagani F. U1-snRNA-mediated rescue of mRNA processing in severe factor VII deficiency. *Blood* 2008, 111:2681–2684.
31. Axelrod FB, Liebes L, Gold-Von Simson G, Mendoza S, Mull J, Leyne M, Norcliffe-Kaufmann L, Kaufmann H, Slangenaupt SA. Kinetin improves

- IKBKAP mRNA splicing in patients with familial dysautonomia. *Pediatr Res* 2011, 70:480–483.
32. Hims MM, Ibrahim EC, Leyne M, Mull J, Liu L, Lazaro C, Shetty RS, Gill S, Gusella JF, Reed R, et al. Therapeutic potential and mechanism of kintin as a treatment for the human splicing disease familial dysautonomia. *J Mol Med* 2007, 85:149–161.
  33. Anderson SL, Qiu J, Rubin BY. EGCG corrects aberrant splicing of IKAP mRNA in cells from patients with familial dysautonomia. *Biochem Biophys Res Commun* 2003, 310:627–633.
  34. Hartmann L, Neveling K, Borkens S, Schneider H, Freund M, Grassman E, Theiss S, Wawer A, Burdach S, Auerbach AD, et al. Correct mRNA processing at a mutant TT splice donor in FANCC ameliorates the clinical phenotype in patients and is enhanced by delivery of suppressor U1 snRNAs. *Am J Hum Genet* 2010, 87:480–493.
  35. Sanchez-Alcudia R, Perez B, Perez-Cerda C, Ugarte M, Desviat LR. Overexpression of adapted U1snRNA in patients' cells to correct a 5' splice site mutation in propionic acidemia. *Mol Genet Metab* 2011, 102:134–138.
  36. Tanner G, Glaus E, Barthelmes D, Ader M, Fleischhauer J, Pagani F, Berger W, Neidhardt J. Therapeutic strategy to rescue mutation-induced exon skipping in rhodopsin by adaptation of U1 snRNA. *Hum Mutat* 2009, 30:255–263.
  37. Glaus E, Schmid F, Da Costa R, Berger W, Neidhardt J. Gene therapeutic approach using mutation-adapted U1 snRNA to correct a RPGR splice defect in patient-derived cells. *Mol Ther* 2011, 19:936–941.
  38. Nakamura K, Du L, Tunuguntla R, Fike F, Cavalieri S, Morio T, Mizutani S, Brusco A, Gatti RA. Functional characterization and targeted correction of ATM mutations identified in Japanese patients with ataxia-telangiectasia. *Hum Mutat* 2012, 33:198–208.
  39. El-Beshlawy A, Mostafa A, Youssry I, Gabr H, Mansour IM, El-Tablawy M, Aziz M, Hussein IR. Correction of aberrant pre-mRNA splicing by antisense oligonucleotides in  $\beta$ -thalassemia Egyptian patients with IVSI-110 mutation. *J Pediatr Hematol Oncol* 2008, 30:281–284.
  40. Lacerra G, Sierakowska H, Carestia C, Fucharoen S, Summerton J, Weller D, Kole R. Restoration of hemoglobin A synthesis in erythroid cells from peripheral blood of thalassemic patients. *Proc Natl Acad Sci U S A* 2000, 97:9591–9596.
  41. Suwanmanee T, Sierakowska H, Fucharoen S, Kole R. Repair of a splicing defect in erythroid cells from patients with  $\beta$ -thalassemia/HbE disorder. *Mol Ther* 2002, 6:718–726.
  42. Svasti S, Suwanmanee T, Fucharoen S, Moulton HM, Nelson MH, Maeda N, Smithies O, Kole R. RNA repair restores hemoglobin expression in IVS2-654 thalassemic mice. *Proc Natl Acad Sci U S A* 2009, 106:1205–1210.
  43. Vacek MM, Ma H, Gemignani F, Lacerra G, Kafri T, Kole R. High-level expression of hemoglobin A in human thalassemic erythroid progenitor cells following lentiviral vector delivery of an antisense snRNA. *Blood* 2003, 101:104–111.
  44. Xie SY, Li W, Ren ZR, Huang SZ, Zeng F, Zeng YT. Correction of  $\beta$ 654-thalassaemia mice using direct intravenous injection of siRNA and antisense RNA vectors. *Int J Hematol* 2011, 93:301–310.
  45. Anczukow O, Buisson M, Leone M, Coutanson C, Lasset C, Calender A, Sinilnikova OM, Mazoyer S. BRCA2 deep intronic mutation causing activation of a cryptic exon: opening toward a new preventive therapeutic strategy. *Clin Cancer Res* 2012, 18:4903–4909.
  46. Vega AI, Perez-Cerda C, Desviat LR, Matthijs G, Ugarte M, Perez B. Functional analysis of three splicing mutations identified in the PMM2 gene: toward a new therapy for congenital disorder of glycosylation type Ia. *Human Mutat* 2009, 30:795–803.
  47. Friedman KJ, Kole J, Cohn JA, Knowles MR, Silverman LM, Kole R. Correction of aberrant splicing of the cystic fibrosis transmembrane conductance regulator (CFTR) gene by antisense oligonucleotides. *J Biol Chem* 1999, 274:36193–36199.
  48. Nissim-Rafinia M, Aviram M, Randell SH, Shushi L, Ozeri E, Chiba-Falek O, Eidelman O, Pollard HB, Yankaskas JR, Kerem B. Restoration of the cystic fibrosis transmembrane conductance regulator function by splicing modulation. *EMBO Rep* 2004, 5:1071–1077.
  49. Gurvich OL, Tuohy TM, Howard MT, Finkel RS, Medne L, Anderson CB, Weiss RB, Wilton SD, Flanigan KM. DMD pseudoexon mutations: splicing efficiency, phenotype, and potential therapy. *Ann Neurol* 2008, 63:81–89.
  50. Taniguchi-Ikeda M, Kobayashi K, Kanagawa M, Yu CC, Mori K, Oda T, Kuga A, Kurahashi H, Akman HO, DiMauro S, et al. Pathogenic exon-trapping by SVA retrotransposon and rescue in Fukuyama muscular dystrophy. *Nature* 2011, 478:127–131.
  51. David A, Srirangalingam U, Metherell LA, Khoo B, Clark AJ. Repair of aberrant splicing in growth hormone receptor by antisense oligonucleotides targeting the splice sites of a pseudoexon. *J Clin Endocrinol Metab* 2010, 95:3542–3546.
  52. Brasil S, Vieceilli HM, Meili D, Rassi A, Desviat LR, Perez B, Ugarte M, Thony B. Pseudoexon exclusion by antisense therapy in 6-pyruvoyl-tetrahydropterin synthase deficiency. *Hum Mutat* 2011, 32:1019–1027.
  53. Osorio FG, Navarro CL, Cadinanos J, Lopez-Mejia IC, Quiros PM, Bartoli C, Rivera J, Tazi J, Guzman G, Varela I, et al. Splicing-directed therapy in a new mouse model of human accelerated aging. *Sci Transl Med* 2011, 3:106ra107.

54. Fong LG, Vickers TA, Farber EA, Choi C, Yun UJ, Hu Y, Yang SH, Coffinier C, Lee R, Yin L, et al. Activating the synthesis of progerin, the mutant prelamin A in Hutchinson-Gilford progeria syndrome, with antisense oligonucleotides. *Hum Mol Genet* 2009, 18:2462–2471.
55. Mancini C, Vaula G, Scalzitti L, Cavalieri S, Bertini E, Aiello C, Lucchini C, Gatti RA, Brussino A, Brusco A. Megalencephalic leukoencephalopathy with subcortical cysts type 1 (MLC1) due to a homozygous deep intronic splicing mutation (c.895-226T>G) abrogated in vitro using an antisense morpholino oligonucleotide. *Neurogenetics* 2012, 13:205–214.
56. Perez B, Rincon A, Jorge-Finnigan A, Richard E, Merinero B, Ugarte M, Desviat LR. Pseudoexon exclusion by antisense therapy in methylmalonic aciduria (MMAuria). *Human Mutat* 2009, 30:1676–1682.
57. Rincon A, Aguado C, Desviat LR, Sanchez-Alcudia R, Ugarte M, Perez B. Propionic and methylmalonic acidemia: antisense therapeutics for intronic variations causing aberrantly spliced messenger RNA. *Am J Hum Genet* 2007, 81:1262–1270.
58. Sanaker PS, Toompuu M, McClorey G, Bindoff LA. Antisense oligonucleotide corrects splice abnormality in hereditary myopathy with lactic acidosis. *Gene* 2012, 494:231–236.
59. Kollberg G, Holme E. Antisense oligonucleotide therapeutics for iron-sulphur cluster deficiency myopathy. *Neuromuscul Disord* 2009, 19:833–836.
60. Wheeler TM, Lueck JD, Swanson MS, Dirksen RT, Thornton CA. Correction of CIC-1 splicing eliminates chloride channelopathy and myotonia in mouse models of myotonic dystrophy. *J Clin Invest* 2007, 117:3952–3957.
61. Pros E, Fernandez-Rodriguez J, Canet B, Benito L, Sanchez A, Benavides A, Ramos FJ, Lopez-Ariztegui MA, Capella G, Blanco I, et al. Antisense therapeutics for neurofibromatosis type 1 caused by deep intronic mutations. *Human Mutat* 2009, 30:454–462.
62. Rodriguez-Pascau L, Coll MJ, Vilageliu L, Grinberg D. Antisense oligonucleotide treatment for a pseudoexon-generating mutation in the NPC1 gene causing Niemann-Pick type C disease. *Human Mutat* 2009, 30:E993–E1001.
63. Lentz JJ, Jodelka FM, Hinrich AJ, McCaffrey KE, Faris HE, Spalitta MJ, Bazan NG, Duelli DM, Rigo F, Hastings ML. Rescue of hearing and vestibular function by antisense oligonucleotides in a mouse model of human deafness. *Nat Med* 2013, 19:345–350.
64. Davis RL, Homer VM, George PM, Brennan SO. A deep intronic mutation in FGB creates a consensus exonic splicing enhancer motif that results in afibrinogenemia caused by aberrant mRNA splicing, which can be corrected in vitro with antisense oligonucleotide treatment. *Hum Mutat* 2009, 30:221–227.
65. Pros E, Fernandez-Rodriguez J, Benito L, Ravella A, Capella G, Blanco I, Serra E, Lazaro C. Modulation of aberrant NF1 pre-mRNA splicing by kinetin treatment. *Eur J Hum Genet* 2010, 18:614–617.
66. Vetrini F, Tammaro R, Bondanza S, Surace EM, Auricchio A, De Luca M, Ballabio A, Marigo V. Aberrant splicing in the ocular albinism type 1 gene (OA1/GPR143) is corrected in vitro by morpholino antisense oligonucleotides. *Hum Mutat* 2006, 27:420–426.
67. Kalbfuss B, Mabon SA, Misteli T. Correction of alternative splicing of tau in frontotemporal dementia and parkinsonism linked to chromosome 17. *J Biol Chem* 2001, 276:42986–42993.
68. Rodriguez-Martin T, Anthony K, Garcia-Blanco MA, Mansfield SG, Anderton BH, Gallo JM. Correction of tau mis-splicing caused by FTDP-17 MAPT mutations by spliceosome-mediated RNA trans-splicing. *Hum Mol Genet* 2009, 18:3266–3273.
69. Bauman JA, Li SD, Yang A, Huang L, Kole R. Antitumor activity of splice-switching oligonucleotides. *Nucleic Acids Res* 2010, 38:8348–8356.
70. Mercatante DR, Mohler JL, Kole R. Cellular response to an antisense-mediated shift of Bcl-x pre-mRNA splicing and antineoplastic agents. *J Biol Chem* 2002, 277:49374–49382.
71. Villemaire J, Dion I, Elela SA, Chabot B. Reprogramming alternative pre-messenger RNA splicing through the use of protein-binding antisense oligonucleotides. *J Biol Chem* 2003, 278:50031–50039.
72. Wilusz JE, Devanney SC, Caputi M. Chimeric peptide nucleic acid compounds modulate splicing of the bcl-x gene in vitro and in vivo. *Nucleic Acids Res* 2005, 33:6547–6554.
73. Bruno IG, Jin W, Cote GJ. Correction of aberrant FGFR1 alternative RNA splicing through targeting of intronic regulatory elements. *Hum Mol Genet* 2004, 13:2409–2420.
74. Shieh JJ, Liu KT, Huang SW, Chen YJ, Hsieh TY. Modification of alternative splicing of Mcl-1 pre-mRNA using antisense morpholino oligonucleotides induces apoptosis in basal cell carcinoma cells. *J Invest Dermatol* 2009, 129:2497–2506.
75. Shiraishi T, Eysturskarth J, Nielsen PE. Modulation of mdm2 pre-mRNA splicing by 9-aminoacridine-PNA (peptide nucleic acid) conjugates targeting intron-exon junctions. *BMC Cancer* 2010, 10:342.
76. Chang JG, Yang DM, Chang WH, Chow LP, Chan WL, Lin HH, Huang HD, Chang YS, Hung CH, Yang WK. Small molecule amiloride modulates oncogenic RNA alternative splicing to devitalize human cancer cells. *PLoS One* 2011, 6:e18643.

77. Chang WH, Liu TC, Yang WK, Lee CC, Lin YH, Chen TY, Chang JG. Amiloride modulates alternative splicing in leukemic cells and resensitizes Bcr-AblT315I mutant cells to imatinib. *Cancer Res* 2011, 71:383–392.
78. Wang Z, Jeon HY, Rigo F, Bennett CF, Krainer AR. Manipulation of PK-M mutually exclusive alternative splicing by antisense oligonucleotides. *Open Biol* 2012, 2:120–133.
79. Ghigna C, De Toledo M, Bonomi S, Valacca C, Gallo S, Apicella M, Eperon I, Tazi J, Biamonti G. Pro-metastatic splicing of Ron proto-oncogene mRNA can be reversed: therapeutic potential of bifunctional oligonucleotides and indole derivatives. *RNA Biol* 2010, 7:495–503.
80. Izaguirre DI, Zhu W, Hai T, Cheung HC, Krahe R, Cote GJ. PTBP1-dependent regulation of USP5 alternative RNA splicing plays a role in glioblastoma tumorigenesis. *Mol Carcinog* 2012, 51:895–906.
81. Geib T, Hertel KJ. Restoration of full-length SMN promoted by adenoviral vectors expressing RNA antisense oligonucleotides embedded in U7 snRNAs. *PLoS One* 2009, 4:e8204.
82. Passini MA, Bu J, Richards AM, Kinnecom C, Sardi SP, Stanek LM, Hua Y, Rigo F, Matson J, Hung G, et al. Antisense oligonucleotides delivered to the mouse CNS ameliorate symptoms of severe spinal muscular atrophy. *Sci Transl Med* 2011, 3:72ra18.
83. Baughan TD, Dickson A, Osman EY, Lorson CL. Delivery of bifunctional RNAs that target an intronic repressor and increase SMN levels in an animal model of spinal muscular atrophy. *Hum Mol Genet* 2009, 18:1600–1611.
84. Bebee TW, Gladman JT, Chandler DS. Splicing regulation of the survival motor neuron genes and implications for treatment of spinal muscular atrophy. *Front Biosci* 2010, 15:1191–1204.
85. Zhang Z, Kelemen O, van Santen MA, Yelton SM, Wendlandt AE, Sviripa VM, Bollen M, Beullens M, Urlaub H, Luhrmann R, et al. Synthesis and characterization of pseudocantharidins, novel phosphatase modulators that promote the inclusion of exon 7 into the SMN (survival of motoneuron) pre-mRNA. *J Biol Chem* 2011, 286:10126–10136.
86. Coady TH, Lorson CL. Trans-splicing-mediated improvement in a severe mouse model of spinal muscular atrophy. *J Neurosci* 2010, 30:126–130.
87. Shababi M, Glascock J, Lorson CL. Combination of SMN trans-splicing and a neurotrophic factor increases the life span and body mass in a severe model of spinal muscular atrophy. *Hum Gene Ther* 2011, 22:135–144.
88. Mowrer KR, Wolfe MS. Promotion of BACE1 mRNA alternative splicing reduces amyloid  $\beta$ -peptide production. *J Biol Chem* 2008, 283:18694–18701.
89. Edmond V, Brambilla C, Brambilla E, Gazzeri S, Eymin B. SRSF2 is required for sodium butyrate-mediated p21(WAF1) induction and premature senescence in human lung carcinoma cell lines. *Cell Cycle* 2011, 10:1968–1977.
90. Pankratova S, Nielsen BN, Shiraishi T, Nielsen PE. PNA-mediated modulation and redirection of Her-2 pre-mRNA splicing: specific skipping of erbB-2 exon 19 coding for the ATP catalytic domain. *Int J Oncol* 2010, 36:29–38.
91. Wan J, Sazani P, Kole R. Modification of HER2 pre-mRNA alternative splicing and its effects on breast cancer cells. *Int J Cancer* 2009, 124:772–777.
92. Owen LA, Uehara H, Cahoon J, Huang W, Simonis J, Ambati BK. Morpholino-mediated increase in soluble Flt-1 expression results in decreased ocular and tumor neovascularization. *PLoS One* 2012, 7:e33576.
93. Lefave CV, Squatrito M, Vorlova S, Rocco GL, Brennan CW, Holland EC, Pan YX, Cartegni L. Splicing factor hnRNPH drives an oncogenic splicing switch in gliomas. *EMBO J* 2011, 30:4084–4097.
94. Vorlova S, Rocco G, Lefave CV, Jodelka FM, Hess K, Hastings ML, Henke E, Cartegni L. Induction of antagonistic soluble decoy receptor tyrosine kinases by intronic polyA activation. *Mol Cell* 2011, 43:927–939.
95. Uehara H, Cho Y, Simonis J, Cahoon J, Archer B, Luo L, Das SK, Singh N, Ambati J, Ambati BK. Dual suppression of hemangiogenesis and lymphangiogenesis by splice-shifting morpholinos targeting vascular endothelial growth factor receptor 2 (KDR). *FASEB J* 2012, 27:76–85.
96. Krupitza G, Harant H, Dittrich E, Szekeres T, Huber H, Dittrich C. Sodium butyrate inhibits c-myc splicing and interferes with signal transduction in ovarian carcinoma cells. *Carcinogenesis* 1995, 16:1199–1205.
97. Kaida D, Motoyoshi H, Tashiro E, Nojima T, Hagiwara M, Ishigami K, Watanabe H, Kitahara T, Yoshida T, Nakajima H, et al. Spliceostatin A targets SF3b and inhibits both splicing and nuclear retention of pre-mRNA. *Nat Chem Biol* 2007, 3:576–583.
98. Kotake Y, Sagane K, Owa T, Mimori-Kiyosue Y, Shimizu H, Uesugi M, Ishihama Y, Iwata M, Mizui Y. Splicing factor SF3b as a target of the antitumor natural product pladienolide. *Nat Chem Biol* 2007, 3:570–575.
99. Furumai R, Uchida K, Komi Y, Yoneyama M, Ishigami K, Watanabe H, Kojima S, Yoshida M. Spliceostatin A blocks angiogenesis by inhibiting global gene expression including VEGF. *Cancer Sci* 2010, 101:2483–2489.
100. Corrionero A, Minana B, Valcarcel J. Reduced fidelity of branch point recognition and alternative splicing induced by the anti-tumor drug spliceostatin A. *Genes Dev* 2011, 25:445–459.

101. Fan L, Lagisetty C, Edwards CC, Webb TR, Potter PM. Sudemycins, novel small molecule analogues of FR901464, induce alternative gene splicing. *ACS Chem Biol* 2011, 6:582–589.
102. Albert BJ, McPherson PA, O'Brien K, Czaicki NL, Destefino V, Osman S, Li M, Day BW, Grabowski PJ, Moore MJ, et al. Meayamycin inhibits pre-messenger RNA splicing and exhibits picomolar activity against multidrug-resistant cells. *Mol Cancer Ther* 2009, 8:2308–2318.
103. Puppini C, Passon N, Franzoni A, Russo D, Damante G. Histone deacetylase inhibitors control the transcription and alternative splicing of prohibitin in thyroid tumor cells. *Oncol Rep* 2011, 25:393–397.
104. Sumanasekera C, Kelemen O, Beullens M, Aubol BE, Adams JA, Sunkara M, Morris A, Bollen M, Andreadis A, Stamm S. C6 pyridinium ceramide influences alternative pre-mRNA splicing by inhibiting protein phosphatase-1. *Nucleic Acids Res* 2012, 40:4025–4039.
105. Hasegawa M, Miura T, Kuzuya K, Inoue A, Won Ki S, Horinouchi S, Yoshida T, Kunoh T, Koseki K, Mino K, et al. Identification of SAP155 as the target of GEX1A (Herboxidiene), an antitumor natural product. *ACS Chem Biol* 2011, 6:229–233.
106. Cooper C, Guo J, Yan Y, Chooniedass-Kothari S, Hube F, Hamedani MK, Murphy LC, Myal Y, Leygue E. Increasing the relative expression of endogenous non-coding Steroid Receptor RNA Activator (SRA) in human breast cancer cells using modified oligonucleotides. *Nucleic Acids Res* 2009, 37:4518–4531.
107. Zammarchi F, de Stanchina E, Bournazou E, Supakorndej T, Martires K, Riedel E, Corben AD, Bromberg JF, Cartegni L. Antitumorigenic potential of STAT3 alternative splicing modulation. *Proc Natl Acad Sci U S A* 2011, 108:17779–17784.
108. Brambilla C, Folini M, Gandellini P, Daprai L, Daidone MG, Zaffaroni N. Oligomer-mediated modulation of hTERT alternative splicing induces telomerase inhibition and cell growth decline in human prostate cancer cells. *Cell Mol Life Sci* 2004, 61:1764–1774.
109. Renshaw J, Orr RM, Walton MI, Te Poele R, Williams RD, Wanciewicz EV, Monia BP, Workman P, Pritchard-Jones K. Disruption of WT1 gene expression and exon 5 splicing following cytotoxic drug treatment: antisense down-regulation of exon 5 alters target gene expression and inhibits cell survival. *Mol Cancer Ther* 2004, 3:1467–1484.
110. Khoo B, Roca X, Chew SL, Krainer AR. Antisense oligonucleotide-induced alternative splicing of the APOB mRNA generates a novel isoform of APOB. *BMC Mol Biol* 2007, 8:3.
111. Siwkowski AM, Malik L, Esau CC, Maier MA, Wanciewicz EV, Albertshofer K, Monia BP, Bennett CF, Eldrup AB. Identification and functional validation of PNAs that inhibit murine CD40 expression by redirection of splicing. *Nucleic Acids Res* 2004, 32:2695–2706.
112. Graziewicz MA, Tarrant TK, Buckley B, Roberts J, Fulton L, Hansen H, Orum H, Kole R, Sazani P. An endogenous TNF- $\alpha$  antagonist induced by splice-switching oligonucleotides reduces inflammation in hepatitis and arthritis mouse models. *Mol Ther* 2008, 16:1316–1322.
113. Karras JG, Maier MA, Lu T, Watt A, Manoharan M. Peptide nucleic acids are potent modulators of endogenous pre-mRNA splicing of the murine interleukin-5 receptor- $\alpha$  chain. *Biochemistry* 2001, 40:7853–7859.
114. Bottcher-Friebertshauser E, Stein DA, Klenk HD, Garten W. Inhibition of influenza virus infection in human airway cell cultures by an antisense peptide-conjugated morpholino oligomer targeting the hemagglutinin-activating protease TMPRSS2. *J Virol* 2011, 85:1554–1562.
115. Kang JK, Malerba A, Popplewell L, Foster K, Dickson G. Antisense-induced myostatin exon skipping leads to muscle hypertrophy in mice following octaguanidine morpholino oligomer treatment. *Mol Ther* 2011, 19:159–164.
116. Gao Y, Zu T, Low WC, Orr HT, McIvor RS. Antisense RNA sequences modulating the ataxin-1 message: molecular model of gene therapy for spinocerebellar ataxia type 1, a dominant-acting unstable trinucleotide repeat disease. *Cell Transplant* 2008, 17:723–734.
117. Anthony K, Feng L, Arechavala-Gomez V, Guglieri M, Straub V, Bushby K, Cirak S, Morgan J, Muntoni F. Exon skipping quantification by qRT-PCR in Duchenne muscular dystrophy patients treated with the antisense oligomer eteplirsen. *Human Gene Ther Methods* 2012, 23:336–345.
118. Aoki Y, Nakamura A, Yokota T, Saito T, Okazawa H, Nagata T, Takeda S. In-frame dystrophin following exon 51-skipping improves muscle pathology and function in the exon 52-deficient mdx mouse. *Mol Ther* 2010, 18:1995–2005.
119. Cirak S, Arechavala-Gomez V, Guglieri M, Feng L, Torelli S, Anthony K, Abbs S, Garralda ME, Bourke J, Wells DJ, et al. Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study. *Lancet* 2011, 378:595–605.
120. Denti MA, Rosa A, D'Antona G, Sthandier O, De Angelis FG, Nicoletti C, Allocca M, Pansarasa O, Parente V, Musaro A, et al. Body-wide gene therapy of Duchenne muscular dystrophy in the mdx mouse model. *Proc Natl Acad Sci U S A* 2006, 103:3758–3763.

121. Goemans NM, Tulinius M, van den Akker JT, Burm BE, Ekhardt PF, Heuvelmans N, Holling T, Janson AA, Platenburg GJ, Sipkens JA, et al. Systemic administration of PRO051 in Duchenne's muscular dystrophy. *N Engl J Med* 2011, 364:1513–1522.
122. Heemskerk H, de Winter CL, van Ommen GJ, van Deutekom JC, Aartsma-Rus A. Development of antisense-mediated exon skipping as a treatment for duchenne muscular dystrophy. *Ann N Y Acad Sci* 2009, 1175:71–79.
123. McClorey G, Moulton HM, Iversen PL, Fletcher S, Wilton SD. Antisense oligonucleotide-induced exon skipping restores dystrophin expression in vitro in a canine model of DMD. *Gene Ther* 2006, 13:1373–1381.
124. Spitali P, Rimessi P, Fabris M, Perrone D, Falzarano S, Bovolenta M, TrabANELLI C, Mari L, Bassi E, Tuffery S, et al. Exon skipping-mediated dystrophin reading frame restoration for small mutations. *Hum Mutat* 2009, 30:1527–1534.
125. Yin H, Betts C, Saleh AF, Ivanova GD, Lee H, Seow Y, Kim D, Gait MJ, Wood MJ. Optimization of peptide nucleic acid antisense oligonucleotides for local and systemic dystrophin splice correction in the mdx mouse. *Mol Ther* 2010, 18:819–827.
126. Aartsma-Rus A. Overview on DMD exon skipping. *Methods Mol Biol* 2012, 867:97–116.
127. O'Leary DA, Sharif O, Anderson P, Tu B, Welch G, Zhou Y, Caldwell JS, Engels IH, Brinker A. Identification of small molecule and genetic modulators of AON-induced dystrophin exon skipping by high-throughput screening. *PLoS One* 2009, 4:e8348.
128. Goto M, Sawamura D, Nishie W, Sakai K, McMillan JR, Akiyama M, Shimizu H. Targeted skipping of a single exon harboring a premature termination codon mutation: implications and potential for gene correction therapy for selective dystrophic epidermolysis bullosa patients. *J Invest Dermatol* 2006, 126:2614–2620.
129. Muraier EM, Gache Y, Gratz IK, Klausegger A, Muss W, Gruber C, Meneguzzi G, Hintner H, Bauer JW. Functional correction of type VII collagen expression in dystrophic epidermolysis bullosa. *J Invest Dermatol* 2011, 131:74–83.
130. Wein N, Avril A, Bartoli M, Beley C, Chaouch S, Laforet P, Behin A, Butler-Browne G, Mouly V, Krahn M, et al. Efficient bypass of mutations in dysferlin deficient patient cells by antisense-induced exon skipping. *Hum Mutat* 2010, 31:136–142.
131. Dhir A, Buratti E. Alternative splicing: role of pseudoexons in human disease and potential therapeutic strategies. *FEBS J* 2010, 277:841–855.
132. Hua Y, Vickers TA, Baker BF, Bennett CF, Krainer AR. Enhancement of SMN2 exon 7 inclusion by antisense oligonucleotides targeting the exon. *PLoS Biol* 2007, 5:e73.
133. Singh NN, Shishimorova M, Cao LC, Gangwani L, Singh RN. A short antisense oligonucleotide masking a unique intronic motif prevents skipping of a critical exon in spinal muscular atrophy. *RNA Biol* 2009, 6:341–350.
134. Williams JH, Schray RC, Patterson CA, Ayitey SO, Tallent MK, Lutz GJ. Oligonucleotide-mediated survival of motor neuron protein expression in CNS improves phenotype in a mouse model of spinal muscular atrophy. *J Neurosci* 2009, 29:7633–7638.
135. Hastings ML, Berniac J, Liu YH, Abato P, Jodelka FM, Barthel L, Kumar S, Dudley C, Nelson M, Larson K, et al. Tetracyclines that promote SMN2 exon 7 splicing as therapeutics for spinal muscular atrophy. *Sci Transl Med* 2009, 1:5ra12.
136. Anderson ES, Lin CH, Xiao X, Stoilov P, Burge CB, Black DL. The cardiotonic steroid digitoxin regulates alternative splicing through depletion of the splicing factors SRSF3 and TRA2B. *RNA* 2012, 18:1041–1049.
137. Gao Y, Vogt A, Forsyth CJ, Koide K. Comparison of Splicing Factor 3b Inhibitors in Human Cells. *Chem-biochem* 2012.
138. Visconte V, Rogers HJ, Singh J, Barnard J, Bupathi M, Traina F, McMahon J, Makishima H, Szpurka H, Jankowska A, et al. SF3B1 haploinsufficiency leads to formation of ring sideroblasts in myelodysplastic syndromes. *Blood* 2012, 120:3173–3186.

# EXHIBIT 49

## SURVEY AND SUMMARY

# Splice-switching antisense oligonucleotides as therapeutic drugs

Mallory A. Havens<sup>1</sup> and Michelle L. Hastings<sup>2,\*</sup>

<sup>1</sup>Department of Biology, Lewis University, Romeoville, IL 60446, USA and <sup>2</sup>Department of Cell Biology and Anatomy, Chicago Medical School, Rosalind Franklin University of Medicine and Science, North Chicago, IL 60064, USA

Received January 25, 2016; Revised June 02, 2016; Accepted June 02, 2016

### ABSTRACT

**Splice-switching oligonucleotides (SSOs) are short, synthetic, antisense, modified nucleic acids that base-pair with a pre-mRNA and disrupt the normal splicing repertoire of the transcript by blocking the RNA–RNA base-pairing or protein–RNA binding interactions that occur between components of the splicing machinery and the pre-mRNA. Splicing of pre-mRNA is required for the proper expression of the vast majority of protein-coding genes, and thus, targeting the process offers a means to manipulate protein production from a gene. Splicing modulation is particularly valuable in cases of disease caused by mutations that lead to disruption of normal splicing or when interfering with the normal splicing process of a gene transcript may be therapeutic. SSOs offer an effective and specific way to target and alter splicing in a therapeutic manner. Here, we discuss the different approaches used to target and alter pre-mRNA splicing with SSOs. We detail the modifications to the nucleic acids that make them promising therapeutics and discuss the challenges to creating effective SSO drugs. We highlight the development of SSOs designed to treat Duchenne muscular dystrophy and spinal muscular atrophy, which are currently being tested in clinical trials.**

### OVERVIEW

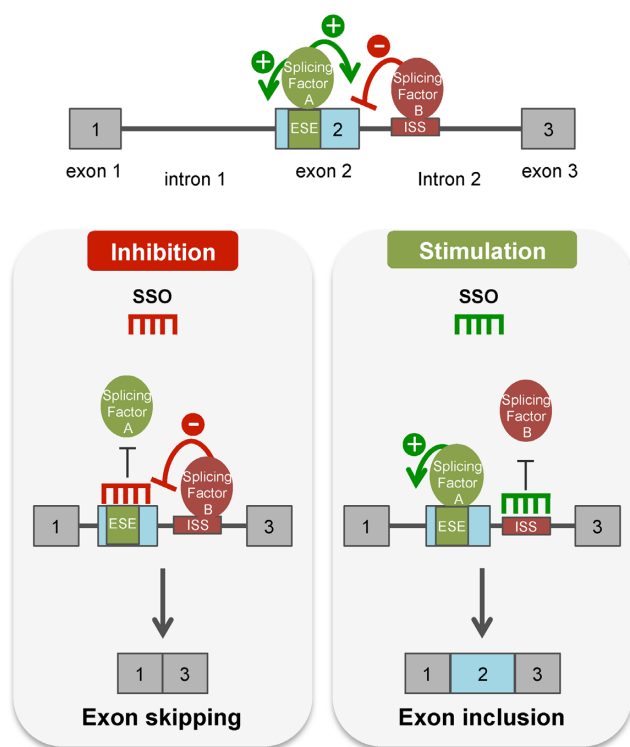
#### Pre-mRNA splicing

Most protein-coding genes are comprised of coding sequences that are interspersed with non-coding sequences. Following gene transcription, these intervening, non-coding RNA sequences, called introns, are removed and the coding RNA sequences, called exons, are ligated to-

gether in a process called pre-mRNA splicing. This splicing gives rise to the final mRNA that is translated into a protein (1). Splicing of each intron involves two sequential trans-esterification reactions. The first reaction releases the 5' exon from the downstream intronic sequence, which forms a lariat structure through an interaction with the branchpoint sequence at the 3' end of the intron (1). The second reaction releases the lariat from the downstream, 3' exon and ligates together the 5' and 3' exons. Pre-mRNA splicing requires precision and accuracy in order to ensure that the proper open reading frame is maintained for efficacious protein production during translation. This high fidelity is achieved, in large part, by sequences and structures within the RNA transcript that direct the binding of splicing proteins that aid in positioning the RNA in a manner that facilitates the correct cleavage and ligation reactions of splicing (2,3). These cleavage reactions occur at conserved sequences called the 5' splice site at the 5' end of an intron and the 3' splice site at the 3' end of an intron. The splice sites are recognized through interactions with a multi-megadalton ribonucleoprotein complex called the spliceosome (4,5). The spliceosome consists of small nuclear RNAs (snRNAs), which form specific RNA–RNA base-pairs with the splice sites, and proteins, all of which function together to direct the spliceosome to the splice sites and position the RNA for the catalytic steps of splicing (1).

In principle, a 5' splice site can splice to any 3' splice site, and the rules that determine the pairing of sites are not entirely clear. It is clear, however, that there can be variability in this process, giving rise to alternative splicing events (6). Indeed, transcriptome sequencing has revealed that splicing of pre-mRNA from most protein-coding genes can occur in a variety of different patterns, giving rise to multiple alternatively spliced isoforms from a single gene (7,8). The regulation of alternative splicing is directed in large part by differential protein binding to cis-acting sequences in the pre-mRNA transcript (6,9). These splicing factor proteins, depending on their function and the location of their binding

\*To whom correspondence should be addressed. Tel: +1 847 578 8517; Fax: +1 847 578 3253; Email: Michelle.hastings@rosalindfranklin.edu



**Figure 1.** Splice-switching oligonucleotides (SSOs) modulate alternative splicing. (top) Diagram of a pre-mRNA transcript with exons depicted as gray boxes and introns as lines. An intronic splicing silencer (ISS, red) and exonic splicing enhancer (ESE, green) are shown bound by a trans-acting inhibitory splicing factor protein (red oval) or stimulatory splicing factor (green oval). These SF proteins either block (–) or promote (+) splicing at splice sites bordering the surrounding exons. (left panel) An SSO that base-pairs to a splicing enhancer sequence creates a steric block to the binding of the stimulatory splicing factor to its cognate enhancer binding site. This block thereby disrupts splicing and results in exon skipping. (right panel) In contrast, an SSO that base-pairs to a splicing silencer sequence element blocks splicing silencer activity by preventing binding of a negatively acting splicing factor. Disruption of the binding of splicing inhibitory proteins to its cognate binding sequence activates splicing at the splice site that is negatively regulated by the silencer element, resulting in exon inclusion.

sites relative to other splicing signals, can either promote or inhibit splicing at a particular site (Figure 1). In the most general terms, a splicing enhancer is defined as a sequence element that, when bound by its cognate protein, promotes the splicing of a nearby exon. In contrast, a splicing silencer is a sequence element that, when bound by its cognate protein, blocks or inhibits splicing at a particular site (Figure 1) (6,10). Splicing silencer and enhancer sequences are further defined by their location in either an exon (e.g. exonic splicing silencer or enhancer) or intron (e.g. intronic splicing silencer or enhancer). The proteins that bind splicing enhancer and silencer elements typically bind in a sequence-specific manner to single-stranded RNA. RNA secondary structures and chromatin structures can also act to influence alternative splicing (11). Much work has been devoted to understanding the splicing code, which aims to explain alternative splicing patterns by the location of cis-acting splicing element sequences, their trans-acting binding proteins, the interactions of these mRNA:protein complexes (mRNP) with surrounding mRNPs and their activity in re-

pressing or enhancing splicing (12,13). An important component of the splicing code is the ability to predict alternative splicing regulation in a tissue-, cell-, condition- and developmental-specific manner and also to predict mutations and other sequence variations that disrupt normal splicing and potentially cause human pathological conditions (14–16). Understanding the splicing code is an important step toward designing strategies for manipulating and switching splicing in a predictable and potentially therapeutic manner.

Alternative splicing expands the diversity of the human proteome and thereby has been hypothesized to contribute to organismal complexity (17,18). For example, though the mouse and human genomes have a similar number of genes, alternative splicing has been estimated to occur in 95–100% of human genes but only 63% of mouse genes (7,8). The prominence of alternative splicing likely explains a certain amount of the functional differences between cell types and suggests that cells can tolerate different isoforms of mRNA and proteins. At the same time, many genetic diseases result from mutations that either cause splicing abnormalities, other errors that alter canonical splicing or reading frameshifts (19–21). Indeed, a recent computational study predicted that the number of mutations that cause disease due to the disruption of splicing is far greater than previously appreciated (14). Given the centrality of splicing in gene expression and its prevalent deregulation in disease, there has been interest in identifying drugs that can specifically modulate splicing in ways that may work to treat disease symptoms. To this end, small molecule discovery and other approaches are being pursued as splice-targeting therapeutics. One particularly promising approach to specifically manipulate splicing at any given site involves the use of short antisense oligonucleotides (ASOs) that base-pair in an antisense orientation to a specific pre-mRNA sequence and, in so doing, modulate splicing by interfering with the normal protein:RNA or RNA:RNA interactions that direct splicing. ASOs that specifically target splicing are referred to here as splice-switching antisense oligonucleotides (SSOs).

### Splice-switching antisense oligonucleotides (SSOs)

ASOs are synthetic molecules comprised of nucleotides or nucleotide analogues that bind to a complementary sequence through Watson–Crick base-pairing. Although all ASO approaches make use of short nucleic acids that specifically base-pair to a targeted sequence, the outcome of such base-pairing depends on the chemistry of the oligonucleotide and the binding location. SSOs are ASOs that are typically 15–30 nucleotides long and designed to base-pair and create a steric block to the binding of splicing factors to the pre-mRNA. In this way, SSO base-pairing to a target RNA alters the recognition of splice sites by the spliceosome, which leads to an alteration of normal splicing of the targeted transcript (Figure 1). Importantly, nucleotides of an SSO are chemically modified so that the RNA-cleaving enzyme RNase H is not recruited to degrade the pre-mRNA-SSO complex (22,23). Thus, SSOs modify splicing without necessarily altering the abundance of the mRNA transcript. The RNase H-resistant features of SSOs

are critical because the goal of SSOs is to alter splicing and not to cause the degradation of the bound pre-mRNA. Modifications to the SSO have also been crucial to stabilize the SSO *in vivo* and improve cellular uptake and release as well as binding affinity.

*Common chemical modifications of splice-switching oligonucleotides.* Key breakthroughs in the chemical design of antisense oligonucleotides have been instrumental in making SSOs a viable therapeutic approach. Natural, unmodified DNA and RNA oligonucleotides are generally unfavorable as therapeutics because they are vulnerable to nuclease degradation in serum and cells and thereby are unstable *in vivo*. Chemical modifications have improved oligonucleotide binding affinity, stability and pharmacodynamic properties. Modifications that improve these qualities involve changes to the phosphate backbone and/or sugar component of the oligonucleotide. These medicinal chemistry efforts have been comprehensively reviewed recently (24–26) and thus, here, we focus our discussion on the specific modifications that have been utilized in the development of SSOs that have shown promise *in vivo* in the treatment of disease/pathological conditions (Table 1).

The phosphorothiate (PS) backbone modification was the first analog to be used in clinical applications and has been incorporated into many SSO designs that are currently being developed as potential therapeutics (Table 1, Figure 2) (27,28). SSOs with a PS backbone modification have modestly reduced binding affinities but have improved stability *in vivo*, with greater nuclease resistance (28). PS ASOs also bind to proteins in plasma, which reduces renal clearance and improves retention, allowing for broad biodistribution, but also increasing the risk of toxicity (28,29).

Oligonucleotides with PS backbone modifications are not resistant to RNase H and thus, to create a steric blocking SSO for splice-switching applications, additional modifications to the molecule are required. ASOs that are fully modified at the 2' sugar position confer RNase H-resistance and are commonly used as SSOs. The most widely used alterations at the 2' position are 2'-O-methyl (2'-OMe) and 2'-O-methoxyethyl (2'-MOE) (Figure 2). Locked nucleic acid (LNA) chemistry, is another modification of the sugar, which involves bridging of the furanose ring (Figure 2) (30). A major benefit of LNA modified SSOs is the elevated binding affinity, which is an important consideration as high binding affinity can allow for the use of shorter SSO sequences. A shorter sequence can reduce the likelihood of binding to an incorrect site as a result of partial sequence complementarity to another sequence and thus lower the risk of unwanted off-target effects. Each of these modifications have been used together (2'-OMe/PS, 2'-MOE/PS; LNA/PS) to successfully target splicing in ways predicted to be therapeutic for a number of different pathological conditions (Table 1).

Phosphorodiamidate morpholinos (PMOs) are another type of modified oligonucleotide that has been used extensively to modify splicing. PMOs have a morpholine ring in place of the furanose ring found in natural nucleic acids and a neutral phosphorodiamidate backbone in place of the negatively charged phosphodiester backbone (Figure 2) (22). The neutral charge of PMOs results in their low bind-

ing of plasma proteins, which improves tolerability *in vivo*. However, they are also rapidly cleared by the kidney and for this reason exhibit lower accumulation in tissues compared to a charged PS backbone (29). As a result, high doses of PMOs may be necessary to elicit a pharmacological response (31,32). A number of approaches have been developed to improve PMO efficacy *in vivo*, as discussed below and shown in Figure 2.

*Delivery routes and mechanisms.* As the ultimate aim of most of the SSO drug designs is to treat a human condition, efficient delivery to cells in the body is imperative. Recent reviews have provided extensive details on the biological basis of ASO access to cells and tissues as well as approaches that are being used to enhance the delivery of ASOs *in vivo* (26,33,34). Thus, here, we highlight some of the approaches that have been used successfully for the delivery of SSOs *in vivo* to alleviate disease phenotypes (Table 1).

A number of different delivery paradigms have been utilized to deliver SSOs to cells *in vivo*, including intraperitoneal (IP), subcutaneous (SC) or intravenous (IV) administration. These methods result in exposure of many peripheral tissues to the oligonucleotide (35–37). Other approaches, such as intramuscular (IM), intratumoral (ITM), subconjunctival (SCJ) or intravitreal (IVI) injection of ASOs have been used to achieve more tissue-specific delivery (Table 1). ASOs do not readily cross the blood brain barrier when administered peripherally (29). However, for therapeutics intended for CNS applications and targets, direct delivery to the cerebrospinal fluid (CSF) by either intracerebroventricular (ICV) or intrathecal (IT) administration has been shown to result in therapeutic doses of SSOs throughout the CNS, though deeper brain regions are more challenging to access (32,38).

The pharmacokinetics, pharmacodynamics and other considerations of central and peripheral administration of ASOs have been expertly reviewed recently (26,39) and we highlight here only a few key points. The effects of a single injection of SSOs on splicing and/or disease have been found to last for up to a year in some tissues when delivered peripherally (40,41) or centrally (32,41,42). This persistent effect of SSOs suggests injections could be minimized. Nonetheless, depending on the condition, SSO therapeutics will likely require repeated dosing. While a repeat dosing regimen may not be a major drawback for the treatment of peripheral tissue, CNS delivery is more invasive and holds greater risk, though repeated IT injections of SSOs in pediatric patients have been shown to be well-tolerated (43). Because of the relative ease of peripheral delivery compared to direct delivery to the CNS, there have been efforts to develop SSO-conjugates that can cross the blood brain barrier (26). However, direct central application has some advantages over peripheral, systemic delivery in that it may allow for lower doses due to the tissue-specific delivery to the CNS and could minimize side effects associated with systemic delivery such as hepatotoxicity (44). Overall, although undoubtedly more invasive and technically challenging than peripheral dosing, a lower dose requirement and less frequent dosing compared to peripheral treatments could lower the amount of drug required for treatment and consequently drug-associated costs. The benefits of limiting

**Table 1.** Splice-switching antisense oligonucleotides with activity *in vivo*. Examples of the most advanced SSO for each target are represented

Condition	Target gene	Stage/Model	SSO	Target (Action)	Route	Ref
Block cryptic/Aberrant splicing caused by mutations						
β-Thalassemia	<i>HBB</i>	mouse	PPMO	intron 2 aberrant 5'ss (correct splicing)	IV	(144)
Fukuyama congenital muscular dystrophy	<i>FKTN</i>	mouse	VPMO	exon 10 aberrant 3'ss; alternative 5'ss; ESE (correct splicing)	IM	(145)
Hutchinson–Gilford progeria	<i>LMNA</i>	mouse	VPMO; 2'-MOE /PS	exon 10 5'ss; exon 11 cryptic 5'ss; exon 11 ESE (block exon 11 splicing)	IV/IP	(146,147)
Leber congenital amaurosis	<i>CEP290</i>	mouse	2'-OMe /PS; AAV	Intron 26 cryptic exon (correct splicing)	IVI	(56)
Myotonic dystrophy	<i>CLCN1</i>	mouse	PMO	exon 7a 3'ss (exon 7a skipping)	IM	(53,148)
Usher syndrome	<i>USH1C</i>	mouse	2'-MOE /PS	exon 3 cryptic 5'ss (correct splicing)	IP	(40)
X-linked agammaglobulinemia	<i>BTX</i>	mouse	PPMO	pseudoexon 4A ESS (pseudoexon skipping)	IV/SC	(149)
Switch alternative splicing						
Alzheimer's disease	<i>LRP8</i>	mouse	2'-MOE /PS	intron 19 ISS (exon 19 inclusion)	ICV	(42)
Autoimmune diabetes susceptibility	<i>CTLA4</i>	mouse	PPMO	exon 2 3'ss (exon skipping)	IP	(150)
Cancer	<i>BCL2L1</i>	mouse	2'-MOE /PS	exon 2 5'ss (alternative 5'ss)	IV/NP	(151)
Cancer	<i>ERBB4</i>	mouse	LNA	exon 26 5'ss (exon skipping)	IP	(152)
Cancer	<i>MDM4</i>	mouse	PMO	exon 6 5'ss (exon skipping)	ITM	(153)
Cancer	<i>STAT3</i>	mouse	VPMO	exon 23 α 3'ss (β 3'ss use)	ITM	(154)
Inflammation	<i>IL1RAP</i>	mouse	2-OMe /PS;LNA	exon 9 ESE (exon skipping)	IV/NP	(155)
Inflammation	<i>TNFRSF1B</i>	mouse	LNA /PS	exon 7 5'ss (exon skipping)	IP	(156)
Neovascularization	<i>FLT1</i>	mouse	PMO	exon 13 5'ss (alternative pA site)	IVI / ITM	(157)
Neovascularization	<i>KDR</i>	mouse	PMO	exon 13 5'ss (alternative pA site)	IVI / SCJ	(158)
Spinal muscular atrophy	<i>SMN2</i>	clinical trials	2'-MOE /PS	intron 7 ISS (exon 7 inclusion)	IT	(43,142)
Correct open reading frame						
cardiomyopathy	<i>MYBPC3</i>	mouse	AAV	Exon 5 and 6 ESEs (exon 5, 6 skipping)	IV	(159)
Cardiomyopathy	<i>TTN</i>	mouse	VPMO	exon 326 ESE (exon skipping)	IP	(160)
Duchenne muscular dystrophy (DMD)	<i>DMD</i>	clinical trials	2'-OMe / PMO	exon 51 ESE (exon skipping)	IV/SC	(46,98)
Nijmegen breakage syndrome	<i>NBN</i>	mouse	VPMO	exon 6/7 ESEs (exon skipping)	IV	(161)
Disrupt open reading frame/Protein function						
Ebola	<i>IL10</i>	mouse	PPMO	exon 4 3'ss (exon skipping)	IP	(162)
Huntington disease	<i>HTT</i>	mouse	2'-OMe /PS	exon 12 skipping	IS	(163)
Hypercholesterolemia	<i>APOB</i>	mouse	2'-OMe /PS	exon 27 3'ss (exon skipping)	IV	(164)
Muscle-Wasting/DMD	<i>MSTN</i>	mouse	PPMO/VPMO/ 2'-OMe	exon 2 ESE (exon skipping)	IV/ IM/ IP	(165,166)
Pompe disease	<i>GYS2</i>	mouse	PPMO	exon 6 5'ss (exon skipping)	IM/IV	(167)
Spinocerebellar ataxia type 3	<i>ATXN3</i>	mouse	2'-OMe /PS	exon 9, 10 skipping	ICV	(168)

AAV (Adeno-associated viral expression of SSO); NP (nano-particle); PPMO (peptide-conjugated phosphorodiamidate morpholino); VPMO (Vivo-PMO).

ICV (intracerebroventricular); IM (intramuscular); IP (intraperitoneal); IS (intrastriatal); IT (intrathecal); ITM (intratumoral); IV (intravenous); IVI (intravitreal); SC (subcutaneous); SCJ (subconjunctival); ISE (Intronic splicing enhancer); ESE (Exonic splicing enhancer); ISS (intronic splicing silencer); pA (polyadenylation).

systemic drug exposure by utilizing tissue-specific ASO delivery approaches apply to other tissues as well, including diseases of the eye, where intravitreal, and subconjunctival delivery have been efficacious in model systems (Table 1).

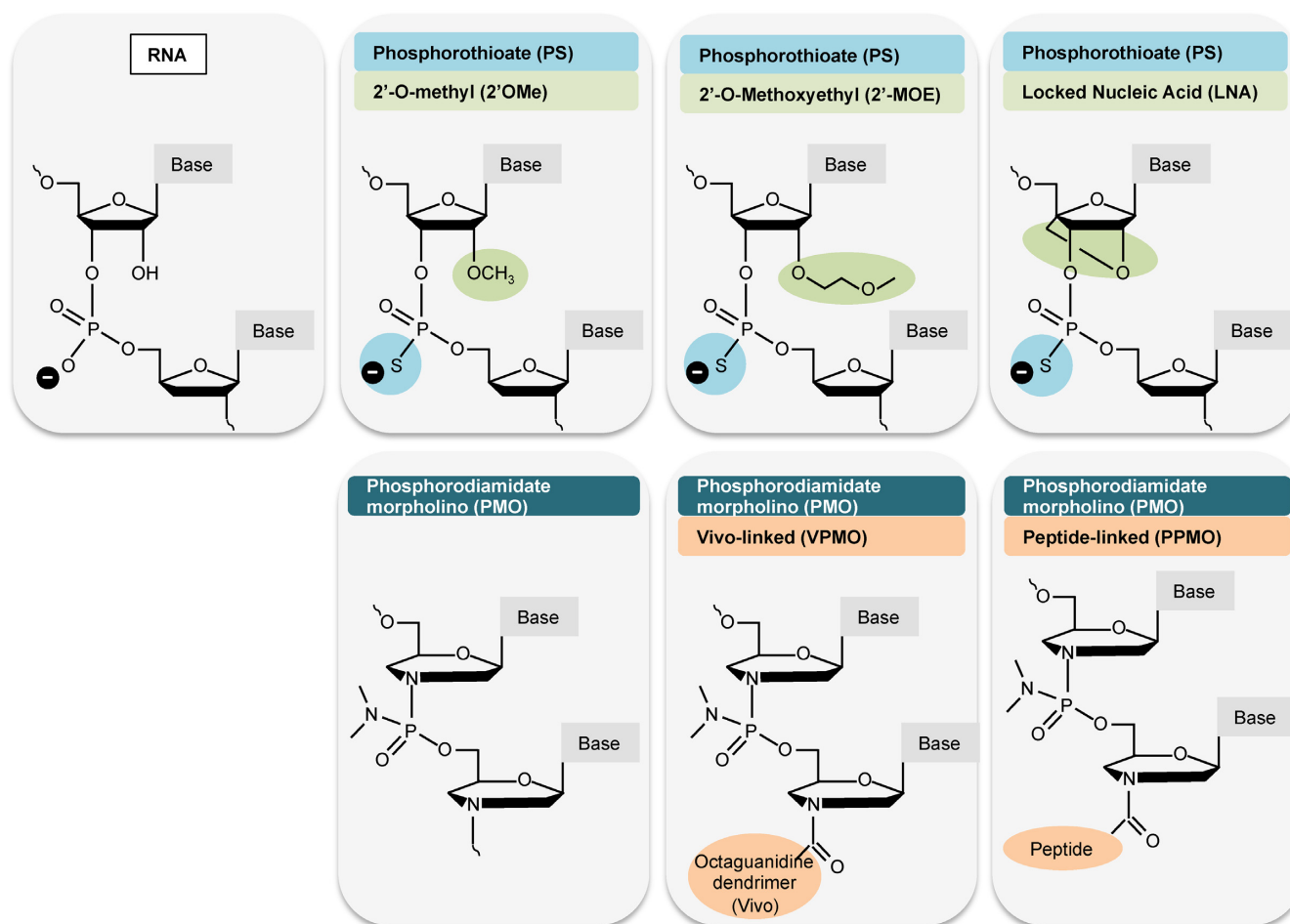
Once injected, ASOs can gain access to cells *in vivo* as naked/unformulated oligonucleotides. ASOs with a charged PS backbone are bound by high and low affinity circulating proteins in the plasma (Figure 3). Protein-binding is thought to be mediated, in part, the up-take of ASOs into cells via vesicular pathways that can either deliver ASOs to lysosomes or release them directly into the cytoplasm through a mechanism that is not well-understood (45) (Figure 3). In contrast, charge-neutral SSOs such as PMOs have reduced protein-binding properties compared to PS backbone-modified ASOs. Nonetheless, studies have demonstrated efficient, non-toxic *in vivo* delivery of both PS-modified SSOs and PMOs by direct injection of the naked oligomer (32,46,47). Once in the cytoplasm, ASOs can move into the nucleus to affect pre-mRNA splicing (48) (Figure 3). Upon entry into cells *in vivo*, ASOs have a long duration of action. SSOs have been shown to affect splicing and disease symptoms for up to a year after a single ICV (32,36) or IP (40) administration in mice.

Although SSOs can be delivered as naked oligonucleotides, modifications, carriers and other approaches offer opportunities to increase efficiency, lower doses and increase tissue-specific delivery, all of which can help to limit toxicity and off-target effects. For example, a number of approaches have been developed to improve PMO efficacy

*in vivo*, including modifications of the 2' position of the sugar with cell-penetrating peptides, or octaguanidine dendrimers (Vivo-morpholinos), which enhance cellular uptake and endosomal release (Table 1, Figure 2) (49–51). Co-administration of PMOs with different agents such as hexose (52), bubble liposomes (53,54) and F127 copolymer (55) have also shown some promise in improving PMO activity *in vivo*. Adeno-associated virus (AAV)-packaged SSO has also been explored for the treatment of disease (Table 1). Though AAV-mediated gene therapy itself is a major therapeutic platform under development for the treatment of a number of diseases, the approach is constrained by cargo size limitations, which makes them less valuable as a therapeutic option for genes with coding sequences greater than 5 kb. A recent study has shown similar efficacy of naked SSOs and AAV-packaged SSOs *in vivo* (56). However, AAV-mediated expression of SSOs may offer advantages of improved delivery to specific cell types and the potential for a longer lasting effect.

## SSO STRATEGIES TO THERAPEUTICALLY MANIPULATE GENE EXPRESSION

SSOs have many of the key attributes that make an ideal drug. They are relatively easy to synthesize and deliver, as they do not strictly require packaging or other delivery intermediates. They are highly target-specific due to their base-pairing requirements, and they exhibit widespread entry into most cell types in the body (29,50,57). SSOs are also well tolerated, particularly in the CNS, and they have



**Figure 2.** Structures of oligonucleotide analogs commonly used in splice switching applications *in vivo*. Modifications that are used in the SSOs presented in Table 1 are depicted. Unmodified RNA is shown for reference. Base refers to unmodified adenine, cytosine, guanine or uracil.

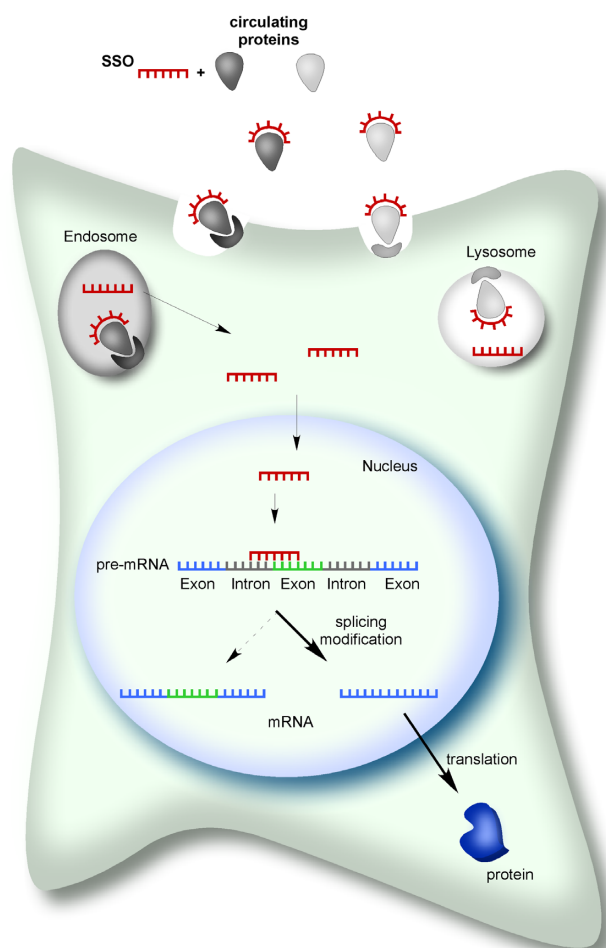
a long-lasting effect *in vivo* (23). Another important feature of SSOs is that they can be easily designed to have any number of different effects on the expression of a gene by either inhibiting or enhancing the use of a specific splice site (20).

Inhibition of splicing using SSOs can be achieved by targeting the molecule to base-pair at a splice site, which will interfere with splicing protein interactions at the location, which is a key step for splicing catalysis. SSO basepairing at a splicing enhancer sequence can also cause splicing inhibition at a particular splice site (Figure 1). Inhibition of a splice site by an SSO offers a way to block a cryptic splice site that is created by a genetic mutation or to switch or modulate alternative splicing patterns in a manner that are predicted to be therapeutic. Inhibition of splicing at a particular site can also be utilized to restore the reading frame of an mRNA by skipping out an exon that either has a premature termination codon created by a mutation or a deletion resulting in a frameshift. In these cases, the exclusion of an exon from the mRNA restores the mRNA reading frame, albeit producing a shortened version of the protein with an internal deletion. SSO-induced exon skipping can also be used to create a frame-shift in an mRNA in order to down-regulate protein production from a gene or to eliminate unwanted or pathological sequences from a protein.

Each of these strategies is being pursued for the treatment of disease and has been shown to be efficacious *in vivo* for a number of different disease models and we present examples of some of the more advanced studies with SSOs for specific genes in Table 1.

SSOs can also be designed to activate or enhance splicing at a particular site. For this, SSOs are often targeted to base-pair at a *cis*-acting splicing silencer sequence, thereby blocking the binding of the associated trans-acting inhibitory protein factor (Figure 1). In this way, SSOs can be used to promote splicing at a splice site that has been weakened by a mutation in the region of the splice site sequence itself or by a mutation in a splicing enhancer sequence that promotes the normal use of the site. SSOs can also be designed to disrupt RNA secondary structures, which can function to either enhance or inhibit splicing (58). Though not a focus of this review, ASOs can also be designed to target other RNA processing events in the pathway to mRNA maturation such as polyadenylation (59).

The first demonstration that ASOs could be used to target splicing came from studies of a thalassemia-associated defect in splicing caused by a mutation in the human  $\beta$ -globin gene that creates a cryptic 5' splice site, which is used preferentially over the natural site (60). In this study,



**Figure 3.** Splice-switching antisense oligonucleotides (SSOs) mechanism of action. SSOs can gain entry into cells *in vivo* following injection of a naked/unformulated ASO into the blood or cerebrospinal fluid. SSOs can be bound by circulating proteins and have been proposed to enter into cells by binding to receptors for these proteins on the cell surface. Subsequently, SSOs undergo compartmentalization followed by vesicle release at which point they are free to move into the nucleus, bind pre-mRNA and induce a splicing switch that results in an mRNA that is translated into a protein isoform in the cytoplasm.

Dominski and Kole demonstrated that a 2'-OMe SSO, designed to base-pair to the region encompassing the cryptic splice site, blocks splicing at the site and redirects splicing to the correct splice site. Since these early studies, targeting splicing with SSOs has been used as a tool to identify cis-acting splicing elements and to modify splicing in ways that are designed to be therapeutic in disease (20). Advances in SSO-based therapeutics and their comparisons to other therapeutic platforms have been extensively reviewed (23,26,39,61–67). Many SSO strategies have now been demonstrated to be effective in modulating splicing in animal models of human disease and some have entered clinical trials (Table 1). Results from tests of splice-switching ASOs in humans were first reported in 2007 for the treatment of Duchenne Muscular Dystrophy (DMD) (65). The most advanced SSOs are now in Phase 3 clinical trials for

the treatment of DMD and another pediatric genetic disorder, Spinal Muscular Atrophy (SMA) (Tables 1 and 2).

## SPLICE-MODIFYING ANTISENSE APPROACHES IN HUMANS

### SSOs for the treatment of Duchenne Muscular Dystrophy (DMD)

DMD is an X-linked neuromuscular disorder that affects 1:5000-10 000 male births (68–70), and is caused by mutations in the *DMD* gene, which codes for dystrophin protein. Dystrophin is an important structural protein in muscle cells that anchors proteins from the internal cytoskeleton to those in the fiber membrane (71). Approximately 70% of DMD mutations are deletions of exons that disrupt the mRNA reading frame and create premature termination codons that produce truncated and usually non-functional dystrophin protein. This lack of functional dystrophin results in progressive muscle weakness beginning typically before the age of 6, followed by loss of ambulation by the age of 12. Death usually occurs in the second decade of life and results from complications related to failure of the respiratory muscles, though most patients also develop cardiomyopathy, which is the primary cause of death in up to 30% of patients (72).

Becker Muscular Dystrophy (BMD) is also caused by mutations in the *DMD* gene and has similar symptoms to DMD but with later onset and slower progression. This difference in phenotypes is related to the type of DMD mutation. DMD mutations resulting in BMD do not disrupt the reading frame and thus produce an altered dystrophin protein with sufficient functionality to ameliorate the severity of disease symptoms (73,74). This spectrum of disease severity, which is driven by the nature of the mutations, gave rise to the idea that inducing exon skipping to correct the reading frame of mRNA from mutated *DMD* could be an effective way to produce a BMD-type dystrophin protein that would partially compensate for the loss of full-length protein and ameliorate the symptoms of DMD (Figure 3). SSOs targeting different exons would allow reading frame correction of over 50% of deletions and 22% of duplications reported in the Leiden DMD-mutation Database (<http://www.dmd.nl/>). In order for an SSO drug for DMD to treat a greater number of patients with different DMD mutations, the use of cocktails containing multiple different SSOs targeting different exons is also a possibility, and could allow for the correction of more than 90% of patient mutations by a frame-correcting exon skipping therapy (75,76).

One of the first demonstrations that SSOs could be used to modulate splicing of a DMD exon involved the use of an SSO to inhibit splicing of exon 19 from a minigene-derived pre-mRNA in an *in vitro* splicing assay (77). This study was followed by reports that SSO-induced exon skipping could be achieved in cells from a mouse model of DMD and in DMD patient-derived cells in culture (78,79). Subsequently, numerous groups tested 2'-OMe and PMO SSOs in mouse models of DMD, and found that exon skipping could be effectively induced by the different SSOs administered by intramuscular, subcutaneous or intravenous injection (55,80–82). Furthermore, weekly injections of SSO, for 7 weeks to 6

**Table 2.** Clinical trials for Eteplirsen, Kyndrisa and Nusinersen

Trial number	Start	End	Age	n	Status	Additional information	Design*	Phase
<b>Eteplirsen<sup>TM</sup> - Duchenne Muscular Dystrophy</b>								
NCT00159250	Oct '07	Mar '09	10–17 yr	7	Completed	non-ambulatory	single blind	1/2
NCT00844597	Jan '09	Dec '10	5–15 yr	19	Completed	25 m unaided walk	open label	1/2
NCT01396239	Jul '11	Jun '12	7–13 yr	12	Completed	200–400 m 6MWD	placebo control	2
NCT01540409	Feb '12	Sep '16	7–13 yr	12	Active	01396239 extension	open label	2
NCT02255552	Sep '14	May '19	7–16 yr	160	Recruiting	>300 m 6MWD	open label, untreated control	3
NCT02286947	Oct '14	Sep '17	7–21 yr	20	Active	non-ambulatory or ≤300m 6MWD	open label	2
NCT02420379	Mar '15	Feb '18	4–6 yr	40	Recruiting		open label	2
<b>Kyndrisa<sup>TM</sup> - Duchenne Muscular Dystrophy</b>								
NCT01910649	Mar '08	Dec '16	5–16 yr	12	Terminated		open label	1/2
NCT01128855	Jul '10	Oct '11	≥9 yr	20	Completed	non-ambulatory	placebo control	1
NCT01153932	Sep '10	Sep '12	≥5 yr	53	Completed	>75 m 6MWD	placebo control	2
NCT01254019	Dec '10	Jun '13	≥5 yr	186	Completed	>75 m 6MWD	placebo control	3
NCT01480245	Sep '11	Mar '14	≥5 yr	233	Terminated	01254019, 01153932 extension	open label	3
NCT01462292	Oct '11	Nov '13	≥5 yr	51	Completed	>75 m 6MWD	placebo control	2
NCT01803412	May '13	Jun '17	≥5 yr	67	Terminated	01480245, 01462292, 01254019 extension	open label	3
NCT02636686	Dec '15	Jan '18	5–80 yr	220	Terminated	extension, ineligible for other trials	open label	3
<b>Nusinersen<sup>TM</sup> - Spinal Muscular Atrophy</b>								
NCT01494701 (CS1)	Nov '11	Jan '13	2–14 yr	28	Completed	Type 2/3 SMA	open label	1
NCT01703988 (CS2)	Oct '12	Jan '15	2–15 yr	34	Completed	Type 2/3 SMA	open label	1/2
NCT01780246 (CS10)	Jan '13	Feb '14	2–15 yr	18	Completed	CS1 extension	open label	1
NCT01839656	May '13	Nov '16	0–210 d	20	Active	Type 1 SMA	open label	2
NCT02052791 (CS12)	Jan '14	Jan '17	Any	52	Active	CS2, CS10 extension	open label	1
NCT02193074 (ENDEAR)	Jul '14	Jul '17	0–210 d	111	Recruiting	Type 1 SMA	sham control	3
NCT02292537 (CHERISH)	Nov '14	Jun '17	2–12 yr	117	Active	Type 2 SMA, non-ambulatory	sham control	3
NCT02386553 (NURTURE)	May '15	Apr '20	0–6 wk	25	Recruiting	Pre-symptomatic, Type 1 SMA	open label	2
NCT02462759 (EMBRACE)	Jun '15	Oct '17	Any	21	Active	ineligible for ENDEAR or CHERISH	sham control	2
NCT02594124 (SHINE)	Nov '15	Feb '20	13 mo–21 yr	274	Active	ENDEAR, CHERISH, CS12 extension	open label	3

\*Unless otherwise noted, open label trials were non-randomized and non-controlled and placebo and sham-controlled studies were double-blind and randomized.

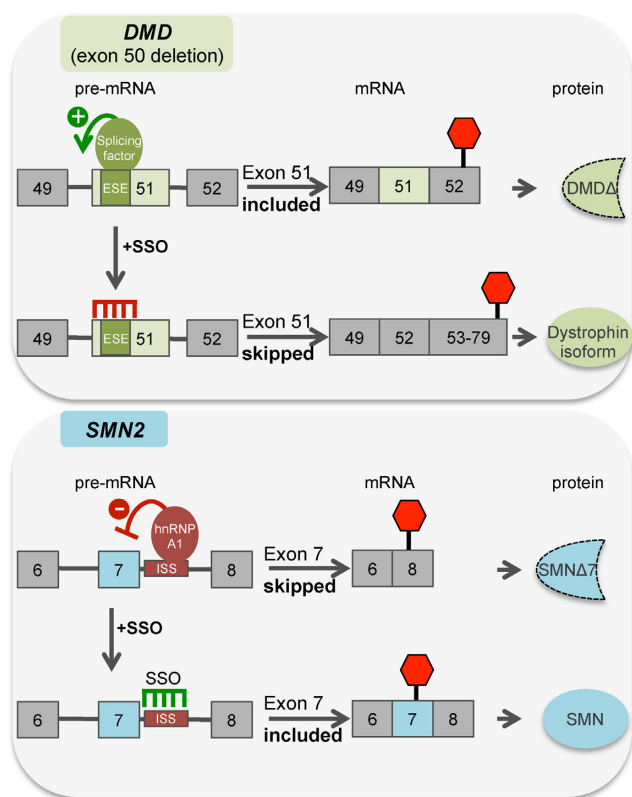
Extension indicates patients participated in a previous study. 6MWD, 6-min walk distance; Yr, years; Mo, months; wk, weeks; d, days.

months, proved to be well tolerated and resulted in enough dystrophin protein to improve muscle function in dystrophic mice (83,84). SSO cocktails aimed at skipping multiple exons for reading frame correction have also been tested and shown to be effective in mice (85) and dogs (31), an advancement that could benefit future clinical development in humans (76).

The promising results in animal models of DMD led to the initiation of clinical trials of SSOs in DMD patients. The first SSO to be tested in a clinical trial for the treatment of DMD was a 31-mer oligodeoxynucleotide phosphorothioate (DNA/PS), which was administered by intravenous injection (86). Though exon skipping and an increase in dystrophin was observed in muscle biopsy tissue, this DNA/PS SSO may have also made dystrophin RNA a substrate for RNase H-targeted degradation decreasing its effectiveness. Today, SSOs targeting numerous DMD exons (8,35,43–45,50,52–55) are being developed as therapies. SSOs that induce skipping of exon 44 (PRO044, BMN 044), exon 45 (SRP-4045, BMN 045), exon 51 (Eteplirsen, Kyndrisa) and exon 53 (SRP-4053, BMN 053) have advanced to clinical trials <http://investorrelations.sarepta.com/phoenix.zhtml?c=64231&p=irol-newsArticle&ID=2007537> (67,87,88) (Table 2). Skipping of exon 51 would benefit the most DMD patients, as ~13% have a frame-shift that could be corrected by the exclusion of this exon from the mRNA (<http://www.dmd.nl/>) (89) (Figure 4). This exon was first tested as a target for SSO-mediated skipping in patient cells lines via retroviral expression of an exon 51 antisense sequence (90). Following a systematic study in human cells to evaluate the optimal chemistry and sequences for a DMD SSO (91), two SSO approaches were pursued. Both SSOs are designed to base-pair in the same DMD RNA target region but they have different lengths and

chemical modifications. Both of these SSOs have been tested in clinical trials to treat DMD. Eteplirsen (AVI-4658) is being developed by Sarepta Therapeutics and Kyndrisa<sup>TM</sup> (Drisapersen/PRO051/GSK2402968), was pursued by Biomarin (formerly developed by Prosensa and GlaxoSmithKline) though has recently been withdrawn from development, as detailed below.

Eteplirsen is a 30-nucleotide, PMO targeting DMD exon 51 for skipping. Studies in mice and non-human primates demonstrated the safety and tolerability of the drug (92–94). Treatment with the SSO was also concluded to be safe and capable of causing exon 51 skipping and increasing dystrophin protein expression in clinical trials in humans when delivered either by intramuscular or intravenous injection (Table 2) (47,95,96). A longitudinal study of 12 patients treated with Eteplirsen for four years concluded that patients receiving the drug had a statistically significant increase on a 6-min walk test (6MWT) compared to natural history data from historical control patients with similar mutations that had not been treated with the SSO. This clinical outcome is accompanied by a very modest 0.9% increase in dystrophin protein in Eteplirsen-treated patients compared to untreated DMD patients <http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/PeripheralandCentralNervousSystemDrugsAdvisoryCommittee/UCM481913.pdf> (46). A recent FDA review of the drug data acknowledged the safety of the drug treatments but questioned the evidence that Eteplirsen treatment benefited patients <http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/PeripheralandCentralNervousSystemDrugsAdvisoryCommittee/UCM481912.pdf>. Specifically, the FDA ques-



**Figure 4.** Schematic representation of disease associated splicing in DMD (top panel) and SMA (bottom panel) and the SSO targeting strategy used to therapeutically switch splicing for the treatment of the disease. Boxes are exons and horizontal lines are introns. Splicing regulatory sequences and protein regulators are noted. ESE, exonic splicing enhancer; ISS, intronic splicing silencer N1. SMN $\Delta$ 7 refers to a form of SMN lacking amino acids encoded by exon 7. Dystrophin $\Delta$  refers to a form of dystrophin truncated after amino acids encoded by exon 52 before encountering a premature termination codon in exon 52. Dystrophin isoform refers to a form of the Dystrophin protein encoded by mRNA lacking exons 50 and 51. The position of the stop codon is indicated by a red hexagon.

tioned the use of natural history as the control, as the patients in that group may have had more severe symptoms than those enrolled in the clinical trial as clinical trial patients were often required to be ambulatory (46)(Table 2). Additionally, the increases in the 6MWT in the treated patients were within the range of the normal progression of the disease. Indeed, independent statistical review and analysis of the data by the FDA concluded that the comparison of Eteplirsen with historical controls was not statistically interpretable. In light of this evidence, an independent FDA review panel declined to recommend Eteplirsen for approval. The FDA is expected to announce its decision on the drug in 2016. Many of the issues holding back the advancement of Eteplirsen are related to the small patient sample size available for clinical trials and the variable, progressive nature of the disease, which make analysis of statistical and clinical significance challenging.

Kyndrisa is a 20-nucleotide, 2'-O-methylphosphorothioate oligomer (2'OMePS) (97). The sequence of Kyndrisa is similar to Eteplirsen, though Eteplirsen is longer, with an additional eight and two nucleotides

on its 5' and 3' ends, respectively. Initial clinical trials found that intramuscular injection resulted in exon 51 skipping and a modest increase in dystrophin protein (97). However, repeated, systemic administration of the drug did not result in dystrophin protein above the range found in untreated patients. Following 25 weeks in a Phase 2 clinical trials, during which DMD patients were treated by subcutaneous injection with Kyndrisa, patients receiving the SSO showed consistent improvements in the 6MWT when compared with patients receiving placebo or intermittent treatment (98,99). However, after 49 weeks of treatment, the difference between Kyndrisa-treated and placebo groups was not statistically significant. Follow-up studies in a larger patient population also did not show a statistically significant improvement in the 6MWT after 48 weeks of treatment. In addition, unlike Eteplirsen, which was well-tolerated by patients, Kyndrisa treatment resulted in adverse events in several organ systems including life-threatening thrombocytopenia. The FDA recently denied an application to approve the drug for DMD, concluding that the drug was not ready for approval and recommended additional placebo-controlled clinical trials, some of which are on-going (Table 2) (<http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/PeripheralandCentralNervousSystemDrugsAdvisoryCommittee/UCM473737.pdf>). An application for marketing approval to the the European Medicines Agency was recently withdrawn and Biomarin programs developing similar SSOs to target exon 44, 45 and 53 skipping have been discontinued (<http://investors.bmrn.com/releasedetail.cfm?ReleaseID=973536>). Biomarin has announced plans to invest in the development of next generation oligonucleotides that will overcome the safety issues associated with Kyndrisa.

The development of an SSO for the treatment of DMD has been challenging and several caveats must be considered when interpreting the clinical results for the SSO DMD trials to date and evaluating the potential of SSO therapies for DMD (100,101). First, the two SSO drugs being tested in DMD patients differ in their chemistries which likely influences the pharmacodynamics and clinical outcomes and may present unique challenges to delivery and dosing. Second, the variable nature of the disease makes it difficult to assess cohort results (102). Third, rare diseases such as DMD have a small patient population available for clinical trials, which make statistical analysis problematic. Fourth, restored dystrophin protein is truncated and semi-functional and therefore, at best, the clinical outcome is conversion to the BMD phenotype. Finally, the timing of treatment and cellular targeting in the clinical trials to date are likely not optimal. Many human muscle fibers are formed embryonically starting at approximately week 11 of gestation and most muscle cells express membrane-associated dystrophin by gestational week 22 (103). Therefore, improving dystrophin expression may be more effective if initiated at an earlier developmental stage. Indeed, studies in mice suggest that SSO-based treatment of DMD symptoms must occur early in pathology, as treatment later in life was not effective at ameliorated disease phenotype

(104,105). In addition, skeletal muscles are not the only tissues impacted by DMD. As mentioned previously, most DMD patients die from a combination of respiratory and cardiac failure. Both 2'-OMe and PMO modifications on ASOs have been found to result in low efficiency in cardiac muscles (57,83). Therefore, additional modifications to the SSOs may be required to target cardiac tissue. To this end, peptide-conjugated PMOs have been developed that improve DMD exon skipping in cardiac tissue (49,57). Although more development is clearly necessary, the progress to date with SSO therapeutics for DMD is substantial and demonstrates that a disease treatment that redirects splicing can be safe and may be therapeutically beneficial to some patients.

### An SSO therapeutic for spinal muscular atrophy

Splice-switching ASO therapeutics are also in clinical trials for the treatment of SMA, an autosomal-recessive disease characterized by motor neuron degeneration that leads to progressive muscle weakness and, in severe cases, respiratory failure and death (106). SMA is the most common genetic cause of infant mortality and affects ~1:10 000 live births with ~1:50 carrier frequency (107–109). Studies on the natural history of infants with the most severe forms of the disease, SMA Type 1, found that 50% had died or were on permanent ventilation by 6.1–13.5 months of age and 80% had these outcomes by 18 months of age (110–112). The severity of SMA warrants an aggressive approach to therapeutic development for a disease that so far has no FDA-approved treatment.

SMA is caused by insufficient production of the protein SMN, a highly conserved and ubiquitously expressed protein involved in pre-mRNA splicing. In humans, SMN protein is produced from two different genes, *SMN1* and *SMN2*, which arose from a duplication at chromosome 5q where the genes are located. Most SMN protein in the cell is produced from the *SMN1* gene. The lack of SMN protein production from *SMN2*, despite the fact that *SMN1* and *SMN2* are nearly identical, is due to the fact that *SMN2* has a single nucleotide C>T difference in exon 7 compared to *SMN1*, which disrupts splicing and results in skipping of exon 7 in most *SMN2* mRNA transcripts (Figure 4) (113–117). This *SMN2* exon 7-skipped mRNA isoform (SMN $\Delta$ 7) codes for an SMN protein isoform that is unstable and does not function in the same manner as the full-length SMN isoform (118,119). People with SMA do not have a functional version of *SMN1* most often due to deletion of part of the gene (120) and the small amount of full-length SMN produced from the limited amount of fully-spliced *SMN2* mRNA cannot fully compensate for the loss of *SMN1* (113). However, the intrinsic instability and variability of the 5q chromosomal region, which is responsible for the high incidence of *SMN1* deletion, can also give rise to genomes with multiple copies of *SMN2*, and a higher *SMN2* copy number is inversely correlated with disease severity (110,121–123). Patients with the most severe form of SMA (Type 1), have the fewest copies of *SMN2*, and most often die in the first few months of life, whereas those with a high *SMN2* copy-number have a less severe form of the disease (Type 2, 3, 4) (110,124,125). Thus, *SMN2* is a

clear genetic modifier of SMA, and consequently elevating *SMN2* expression and full-length SMN protein production from the gene has been a major focus of SMA therapeutic strategies. Because skipping of exon 7 is the major cause of the low SMN production from *SMN2*, SSOs have been heavily studied as a means to target exon 7 splicing and promote its inclusion to improve SMN protein expression (Figure 4).

The first SSOs used to increase *SMN2* exon 7 splicing were targeted to the 3' splice site of exon 8 and resulted in an increase in the use of the 3' splice site of exon 7 and, thereby, more exon 7 inclusion (126). This study was followed by reports that SSOs could be used to increase exon 7 splicing by blocking putative splicing silencer elements surrounding exon 7 (127–129). Since this early work, numerous ASO-based approaches have been shown to effectively increase inclusion of exon 7 in *SMN2* mRNA (23,130). Much of the current work on SSOs for SMA is focused on optimizing the length and the oligonucleotide target sequence as well as testing different backbone modifications to improve the efficacy and pharmacokinetics of the SSO (131–135). Some of the most widely-studied SSOs to date base-pair and block recognition of a splicing silencer element called intronic splicing silencer-N1 (ISS-N1), which is located in intron 7 of the *SMN2* gene (136). The ISS-N1 RNA sequence is recognized by hnRNPA1/A2, which, upon binding, repressed *SMN2* exon 7 splicing (137). SSOs that base-pair with ISS-N1 block hnRNPA1/A2 and relieve the splicing inhibition, which increases exon 7 inclusion full length SMN production (41) (Figure 4). ISS-N1 targeting by SSOs has been shown to dramatically increase the survival of SMA mice (36,131,132,138–141).

The most advanced splice-modulating SSO for the treatment of SMA is Nusinersen (formerly ISIS-SMN<sub>Rx</sub>, ASO-10-27, ISIS 396443), first designed and reported by Krainer and colleagues in 2008 and now in Phase 3 clinical trials (Table 2 and Figure 4) (137). Ionis Pharmaceuticals (formerly Isis Pharmaceuticals) is developing the ASO in conjunction with Biogen Idec. Nusinersen is an 18-nucleotide 2'-MOE SSO with a fully modified PS backbone. Pre-clinical studies in SMA mice and non-human primates validated Nusinersen functionality, deliverability and safety (32,36,139).

Nusinersen was first administered to humans in 2011 in a Phase 1 open-label safety, tolerability and dosing study in Type 2 and Type 3 SMA children aged 2–14 years of age (Table 2). The SSO was delivered directly to the CNS by IT injection via lumbar puncture. Though there were only 28 participants with 6 children in each of three different dose cohorts and 10 children in a fourth dose cohort, the results of this first-in-human study were promising in several aspects. First, the lumbar puncture procedure was found to be feasible, well-tolerated and safe method for repeated IT delivery of the SSO (43). Second, the half-life of the SSO in the cerebrospinal fluid (CSF) was 4–6 months (142). This long half-life is within the tolerated range of feasible repeat IT dosing, which was performed total of three times, once at initial dosing and again at eight days and 9–14 months later (43,142). Finally, the children treated with the highest dose (9 mg) of SSO had a significant increase in SMN protein levels in the CSF and an improvement in clinical assessment measurements (Hammersmith Functional Motor

Scale Expanded and Pediatric Quality of Life Inventory) at 9–14 months after treatment (142). These results, though encouraging, are preliminary and will require confirmation by larger, controlled studies.

To date, 10 clinical trials with Nusinersen have been completed or are on-going and current trials are expected to continue through 2020 (Table 2). Together, these trials have treated infants, children and young adults between the ages of  $\leq 6$  weeks old up to 21 years of age. All SSO treatments have been administered by IT injection by lumbar puncture of either a single or a repeated dose three times over the course of three months. Maintenance doses every 4–6 months are now in place for individuals that have completed initial dosing regimens (<http://Clinicaltrials.gov>). Although no results from Phase 2 or 3 clinical trials have been published yet, recent press releases from Ionis Pharmaceuticals claim that there had been no serious adverse effects related to the drug treatment and some children have been on treatment for more than 46 months (<http://ir.ionispharma.com/phoenix.zhtml?c=222170&p=irol-newsArticle&ID=2061208>). The company further reports that Type 1 SMA children that had been on Nusinersen treatment for the longest had a median event-free age of more than 20 months and 73% of the infants still enrolled in the study were event free, older than 15 months of age, had achieved motor milestones and had increased muscle function including some three year old children from the studies that are not on permanent ventilation and some that are walking, which is unprecedented for Type 1 SMA children (<http://ir.ionispharma.com/phoenix.zhtml?c=222170&p=irol-presentations>) (<http://ir.ionispharma.com/phoenix.zhtml?c=222170&p=irol-newsArticle&ID=2061208>). Although two deaths were reported in children that received the full dosing regimens, this number is less than expected from natural history studies (110). The molecular effects of Nusinersen have also been promising. Ionis reports that analysis of autopsy tissue found that the concentration of Nusinersen in the CNS was greater than the concentration that showed biological activity in animal studies (<http://ir.ionispharma.com/phoenix.zhtml?c=222170&p=irol-newsArticle&ID=2061208>). In addition, there was a higher abundance of full-length *SMN2* mRNA and SMN protein in infants treated with Nusinersen compared to untreated SMA infants.

Although the early company reports from the Phase 2 clinical trials suggest drug efficacy, the results have not yet been published so critical evaluation is not possible. Furthermore, these reports are of results from open-label trials, which are not randomized, well-controlled studies (Table 2). Randomized, double-blind, sham-procedure controlled Phase 3 clinical trials are currently underway to help definitively assess the efficacy and safety of Nusinersen in infants and children with SMA. In addition, there is some question as to whether CNS delivery of Nusinersen will be sufficient as a disease therapeutic, considering the fact that in mouse models of SMA, it has become increasingly clear that restoring SMN in peripheral tissues is important for treatment of the disease in mice (36,140,141). In these studies, systemic ASO delivery to peripheral tissue resulted in greatly improved long-term rescue of SMA phenotypes.

Therefore, it will be important that human clinical trials consider ASO delivery to both the CNS and the periphery. The coming months and years will reveal the full potential of Nusinersen as an SMA therapeutic as the effects of optimized dosing regimens and treatment windows become available from the current clinical trials.

## CONCLUSIONS AND FUTURE PERSPECTIVES

The most developed therapeutic SSO programs are designed to treat two severe pediatric diseases. The SSO drugs for their treatment have different chemistries but all aim to alter splicing to increase functional protein expression from the targeted gene transcript. Eteplirsen and Nusinersen, have been well-tolerated by subjects, which is a major victory for the use of SSOs in the clinic. Outcome measurements of efficacy have been mixed for the DMD SSOs but early results from Nusinersen in SMA trials appear promising (142). It is important to consider the differences between the treatment paradigms for SMA and DMD when comparing their efficacy in the clinic. For example, the SSO for SMA, when effectively targeted, results in the production of the functional, full-length SMN protein. In contrast, the DMD SSO treatments are expected to reduce the severity of the disease to a condition similar to the less devastating BMD by inducing exon skipping and the production of a partially functional protein isoform. Thus, the DMD SSOs may not be expected to be as therapeutically beneficial as the SSO used to treat SMA. Additionally, the SSO drugs in clinical trials (Table 2) have different modifications and are delivered as naked/unformulated oligomers. It is possible and even likely that future drug optimization and delivery will lead to better efficacy. Finally, the DMD trials involve subcutaneous or intravenous administration to target skeletal muscles, whereas the SMA therapeutic is administered by intrathecal injection to specifically target the CNS. The pharmacokinetic profiles of ASOs in the CNS and peripheral organs are distinct, which likely affects the efficacy of an ASO (32,35,143). The fact that clinical trials with SSOs for DMD resulted in statistically significant changes in primary outcome measures suggest that the SSO may cause some exon skipping, but the lack of convincing clinical benefit to patients suggests that more work must be done to improve SSO efficacy by optimizing delivery, dosing or other features of the treatment and or SSO. A similar critical evaluation of Nusinersen awaits further reporting of results from current clinical trials with the drug.

The correction of aberrant gene expression has long been a focus of therapeutic development for the treatment of the human disease. ASOs, and in particular SSOs, offer a treatment approach that allows for specific and defined control of gene expression that can be easily tailored to correct or bypass the effects of a specific mutation. For this reason, the use of SSOs for the manipulation of splicing and gene expression is gaining favor as a drug platform for the treatment of disease. The fact that the mechanism of action of any ASO is known a priori by the nature of its design makes it amenable to rapid and systematic optimization. Methods and modifications that improve SSO and ASO drug profiles, in general, are being actively investigated as this drug platform gains traction and favor in the therapeutics arena.

Efforts to increase cellular uptake *in vivo*, limit off-target effects and gain tissue and cell-specific entry are just some of the aims of creating better antisense drugs. Table 1 provides examples of SSOs that have been shown to be effective *in vivo* for the molecular and, in some cases, functional correction of disease defects, and provides insight into potential SSO-based therapeutics on the horizon.

## ACKNOWLEDGEMENTS

The authors thank Jennifer Chang, Frederic Depreux, Dominik Duelli, Anthony Hinrich, Tiffany Chairuden and Francine Jodelka for comments on the manuscript, and Linda Schwartz for critical comments and editing.

## FUNDING

National Institutes of Health (NIH) [NS069759 and DC012596 to M.L.H.]; Foundation Fighting Blindness; Batten Disease Support and Research Association; Batten Research Alliance; Noah's Hope; Hope for Bridget; and Charlotte and Gwenyth Gray Foundation. The open access publication charge for this paper has been waived by Oxford University Press - NAR.

*Conflict of interest statement.* M.L.H. receives funding from Ionis Pharmaceuticals.

## REFERENCES

- Papasaikas, P. and Valcarcel, J. (2016) The Spliceosome: The ultimate RNA chaperone and sculptor. *Trends Biochem. Sci.*, **41**, 33–45.
- Hang, J., Wan, R., Yan, C. and Shi, Y. (2015) Structural basis of pre-mRNA splicing. *Science*, **349**, 1191–1198.
- Wan, R., Yan, C., Bai, R., Wang, L., Huang, M., Wong, C.C. and Shi, Y. (2016) The 3.8 Å structure of the U4/U6.U5 tri-snRNP: Insights into spliceosome assembly and catalysis. *Science*, **351**, 466–475.
- Brody, E. and Abelson, J. (1985) The “spliceosome”: yeast pre-messenger RNA associates with a 40S complex in a splicing-dependent reaction. *Science*, **228**, 963–967.
- Will, C.L. and Luhrmann, R. (2011) Spliceosome structure and function. *Cold Spring Harb. Perspect. Biol.*, **3**, a003707.
- Lee, Y. and Rio, D.C. (2015) Mechanisms and regulation of alternative Pre-mRNA splicing. *Annu. Rev. Biochem.*, **84**, 291–323.
- Pan, Q., Shai, O., Lee, L.J., Frey, B.J. and Blencowe, B.J. (2008) Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat. Genet.*, **40**, 1413–1415.
- Wang, Z. and Burge, C.B. (2008) Splicing regulation: from a parts list of regulatory elements to an integrated splicing code. *RNA*, **14**, 802–813.
- Barash, Y., Calarco, J.A., Gao, W., Pan, Q., Wang, X., Shai, O., Blencowe, B.J. and Frey, B.J. (2010) Deciphering the splicing code. *Nature*, **465**, 53–59.
- Singh, G., Pratt, G., Yeo, G.W. and Moore, M.J. (2015) The clothes make the mRNA: Past and present Trends in mRNP fashion. *Annu. Rev. Biochem.*, **84**, 325–354.
- Luco, R.F. and Misteli, T. (2011) More than a splicing code: integrating the role of RNA, chromatin and non-coding RNA in alternative splicing regulation. *Curr. Opin. Genet. Dev.*, **21**, 366–372.
- Fu, X.D. (2004) Towards a splicing code. *Cell*, **119**, 736–738.
- Matlin, A.J., Clark, F. and Smith, C.W. (2005) Understanding alternative splicing: towards a cellular code. *Nat. Rev. Mol. Cell Biol.*, **6**, 386–398.
- Xiong, H.Y., Alipanahi, B., Lee, L.J., Bretschneider, H., Merico, D., Yuen, R.K., Hua, Y., Gueroussov, S., Najafabadi, H.S., Hughes, T.R. *et al.* (2015) RNA splicing. The human splicing code reveals new insights into the genetic determinants of disease. *Science*, **347**, 144–151.
- Wang, G.S. and Cooper, T.A. (2007) Splicing in disease: disruption of the splicing code and the decoding machinery. *Nat. Rev. Genet.*, **8**, 749–761.
- Leung, M.K., Xiong, H.Y., Lee, L.J. and Frey, B.J. (2014) Deep learning of the tissue-regulated splicing code. *Bioinformatics*, **30**, i121–i129.
- Gueroussov, S., Gonatopoulos-Pournatzis, T., Irimia, M., Raj, B., Lin, Z.Y., Gingras, A.C. and Blencowe, B.J. (2015) An alternative splicing event amplifies evolutionary differences between vertebrates. *Science*, **349**, 868–873.
- Barbosa-Morais, N.L., Irimia, M., Pan, Q., Xiong, H.Y., Gueroussov, S., Lee, L.J., Slobodeniuc, V., Kutter, C., Watt, S., Colak, R. *et al.* (2012) The evolutionary landscape of alternative splicing in vertebrate species. *Science*, **338**, 1587–1593.
- Chabot, B. and Shkreta, L. (2016) Defective control of pre-messenger RNA splicing in human disease. *J. Cell Biol.*, **212**, 13–27.
- Havens, M.A., Duelli, D.M. and Hastings, M.L. (2013) Targeting RNA splicing for disease therapy. *Wiley Interdiscip. Rev. RNA*, **4**, 247–266.
- Sterne-Weiler, T. and Sanford, J.R. (2014) Exon identity crisis: disease-causing mutations that disrupt the splicing code. *Genome Biol.*, **15**, 201–208.
- Summerton, J. (1999) Morpholino antisense oligomers: the case for an RNase H-independent structural type. *Biochim. Biophys. Acta*, **1489**, 141–158.
- Rigo, F., Seth, P.P. and Bennett, C.F. (2014) Antisense oligonucleotide-based therapies for diseases caused by pre-mRNA processing defects. *Adv. Exp. Med. Biol.*, **825**, 303–352.
- Sharma, V.K. and Watts, J.K. (2015) Oligonucleotide therapeutics: chemistry, delivery and clinical progress. *Future Med. Chem.*, **7**, 2221–2242.
- Saleh, A.F., Arzumanov, A.A. and Gait, M.J. (2012) Overview of alternative oligonucleotide chemistries for exon skipping. *Methods Mol. Biol.*, **867**, 365–378.
- Evers, M.M., Toonen, L.J. and van Roon-Mom, W.M. (2015) Antisense oligonucleotides in therapy for neurodegenerative disorders. *Adv. Drug Deliv. Rev.*, **87**, 90–103.
- Stein, C.A., Subasinghe, C., Shinozuka, K. and Cohen, J.S. (1988) Physicochemical properties of phosphorothioate oligodeoxynucleotides. *Nucleic Acids Res.*, **16**, 3209–3221.
- Eckstein, F. (2014) Phosphorothioates, essential components of therapeutic oligonucleotides. *Nucleic Acid Ther.*, **24**, 374–387.
- Geary, R.S. (2009) Antisense oligonucleotide pharmacokinetics and metabolism. *Expert Opin. Drug Metab. Toxicol.*, **5**, 381–391.
- Campbell, M.A. and Wengel, J. (2011) Locked vs. unlocked nucleic acids (LNA vs. UNA): contrasting structures work towards common therapeutic goals. *Chem. Soc. Rev.*, **40**, 5680–5689.
- Yokota, T., Lu, Q.L., Partridge, T., Kobayashi, M., Nakamura, A., Takeda, S. and Hoffman, E. (2009) Efficacy of systemic morpholino exon-skipping in Duchenne dystrophy dogs. *Ann. Neurol.*, **65**, 667–676.
- Rigo, F., Chun, S.J., Norris, D.A., Hung, G., Lee, S., Matson, J., Fey, R.A., Gaus, H., Hua, Y., Grundy, J.S. *et al.* (2014) Pharmacology of a central nervous system delivered 2'-O-methoxyethyl-modified survival of motor neuron splicing oligonucleotide in mice and nonhuman primates. *J. Pharmacol. Exp. Ther.*, **350**, 46–55.
- Juliano, R.L. (2016) The delivery of therapeutic oligonucleotides. *Nucleic Acids Res.*, doi:10.1093/nar/gkw236.
- Lu, Q.L. and Wu, B. (2012) Systemic delivery of antisense oligomer in animal models and its implications for treating DMD. *Methods Mol. Biol.*, **867**, 393–405.
- Geary, R.S., Norris, D., Yu, R. and Bennett, C.F. (2015) Pharmacokinetics, biodistribution and cell uptake of antisense oligonucleotides. *Adv. Drug Deliv. Rev.*, **87**, 46–51.
- Hua, Y., Sahashi, K., Rigo, F., Hung, G., Horev, G., Bennett, C.F. and Krainer, A.R. (2011) Peripheral SMN restoration is essential for long-term rescue of a severe spinal muscular atrophy mouse model. *Nature*, **478**, 123–126.
- Hung, G., Xiao, X., Peralta, R., Bhattacharjee, G., Murray, S., Norris, D., Guo, S. and Monia, B.P. (2013) Characterization of target mRNA reduction through in situ RNA hybridization in multiple organ systems following systemic antisense treatment in animals. *Nucleic Acid Ther.*, **23**, 369–378.

38. Smith, R.A., Miller, T.M., Yamanaka, K., Monia, B.P., Condon, T.P., Hung, G., Lobsiger, C.S., Ward, C.M., McAlonis-Downes, M., Wei, H. *et al.* (2006) Antisense oligonucleotide therapy for neurodegenerative disease. *J. Clin. Invest.*, **116**, 2290–2296.
39. Southwell, A.L., Skotte, N.H., Bennett, C.F. and Hayden, M.R. (2012) Antisense oligonucleotide therapeutics for inherited neurodegenerative diseases. *Trends Mol. Med.*, **18**, 634–643.
40. Lentz, J.J., Jodelka, F.M., Hinrich, A.J., McCaffrey, K.E., Farris, H.E., Spalitta, M.J., Bazan, N.G., Duelli, D.M., Rigo, F. and Hastings, M.L. (2013) Rescue of hearing and vestibular function by antisense oligonucleotides in a mouse model of human deafness. *Nat. Med.*, **19**, 345–350.
41. Hua, Y., Sahashi, K., Hung, G., Rigo, F., Passini, M.A., Bennett, C.F. and Krainer, A.R. (2010) Antisense correction of SMN2 splicing in the CNS rescues necrosis in a type III SMA mouse model. *Genes Dev.*, **24**, 1634–1644.
42. Hinrich, A.J., Jodelka, F.M., Chang, J.L., Brutman, D., Bruno, A.M., Briggs, C.A., James, B.D., Stutzmann, G.E., Bennett, D.A., Miller, S.A. *et al.* (2016) Therapeutic correction of ApoER2 splicing in Alzheimer's disease mice using antisense oligonucleotides. *EMBO Mol. Med.*, **8**, 328–345.
43. Hache, M., Swoboda, K.J., Sethna, N., Farrow-Gillespie, A., Khandji, A., Xia, S. and Bishop, K.M. (2016) Intrathecal injections in children with spinal muscular atrophy: Nusinersen clinical trial experience. *J. Child Neurol.*, **31**, 899–906.
44. Calias, P., Banks, W.A., Begley, D., Scarpa, M. and Dickson, P. (2014) Intrathecal delivery of protein therapeutics to the brain: a critical reassessment. *Pharmacol. Ther.*, **144**, 114–122.
45. Koller, E., Vincent, T.M., Chappell, A., De, S., Manoharan, M. and Bennett, C.F. (2011) Mechanisms of single-stranded phosphorothioate modified antisense oligonucleotide accumulation in hepatocytes. *Nucleic Acids Res.*, **39**, 4795–4807.
46. Mendell, J.R., Goemans, N., Lowes, L.P., Alfano, L.N., Berry, K., Shao, J., Kaye, E.M., Mercuri, E., Eteplirsens Study, G. and Network, D.M.D.I. (2015) Longitudinal effect of eteplirsens vs. historical control on ambulation in DMD. *Ann. Neurol.*, **79**, 257–271.
47. Cirak, S., Arechavala-Gomez, V., Guglieri, M., Feng, L., Torelli, S., Anthony, K., Abbs, S., Garralda, M.E., Bourke, J., Wells, D.J. *et al.* (2011) Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study. *Lancet*, **378**, 595–605.
48. Lorenz, P., Misteli, T., Baker, B.F., Bennett, C.F. and Spector, D.L. (2000) Nucleocytoplasmic shuttling: a novel in vivo property of antisense phosphorothioate oligodeoxynucleotides. *Nucleic Acids Res.*, **28**, 582–592.
49. Betts, C., Saleh, A.F., Arzumanov, A.A., Hammond, S.M., Godfrey, C., Coursindel, T., Gait, M.J. and Wood, M.J. (2012) Pip6-PMO, A New Generation of Peptide-oligonucleotide Conjugates With Improved Cardiac Exon Skipping Activity for DMD Treatment. *Mol. Ther. Nucleic Acids*, **1**, e38.
50. Moulton, J.D. and Jiang, S. (2009) Gene knockdowns in adult animals: PPMOs and vivo-morpholinos. *Molecules*, **14**, 1304–1323.
51. El Andaloussi, S.A., Hammond, S.M., Mager, I. and Wood, M.J. (2012) Use of cell-penetrating-peptides in oligonucleotide splice switching therapy. *Curr. Gene Ther.*, **12**, 161–178.
52. Han, G., Gu, B., Cao, L., Gao, X., Wang, Q., Seow, Y., Zhang, N., Wood, M.J. and Yin, H. (2016) Hexose enhances oligonucleotide delivery and exon skipping in dystrophin-deficient mdx mice. *Nat. Commun.*, **7**, 10981.
53. Koebis, M., Kiyatake, T., Yamaura, H., Nagano, K., Higashihara, M., Sonoo, M., Hayashi, Y., Negishi, Y., Endo-Takahashi, Y., Yanagihara, D. *et al.* (2013) Ultrasound-enhanced delivery of morpholino with Bubble liposomes ameliorates the myotonia of myotonic dystrophy model mice. *Sci. Rep.*, **3**, 2242.
54. Negishi, Y., Ishii, Y., Shiono, H., Akiyama, S., Sekine, S., Kojima, T., Mayama, S., Kikuchi, T., Hamano, N., Endo-Takahashi, Y. *et al.* (2014) Bubble liposomes and ultrasound exposure improve localized morpholino oligomer delivery into the skeletal muscles of dystrophic mdx mice. *Mol. Pharm.*, **11**, 1053–1061.
55. Lu, Q.L., Rabinowitz, A., Chen, Y.C., Yokota, T., Yin, H., Alter, J., Jadoon, A., Bou-Gharios, G. and Partridge, T. (2005) Systemic delivery of antisense oligonucleotide restores dystrophin expression in body-wide skeletal muscles. *Proc. Natl. Acad. Sci. U.S.A.*, **102**, 198–203.
56. Garanto, A., Chung, D.C., Duijkers, L., Corral-Serrano, J.C., Messchaert, M., Xiao, R., Bennett, J., Vandenberghe, L.H. and Collin, R.W. (2016) In vitro and in vivo rescue of aberrant splicing in CEP290-associated LCA by antisense oligonucleotide delivery. *Hum. Mol. Genet.*, **2016**, ddw118.
57. Moulton, H.M. and Moulton, J.D. (2010) Morpholinos and their peptide conjugates: therapeutic promise and challenge for Duchenne muscular dystrophy. *Biochim. Biophys. Acta*, **1798**, 2296–2303.
58. Peacey, E., Rodriguez, L., Liu, Y. and Wolfe, M.S. (2012) Targeting a pre-mRNA structure with bipartite antisense molecules modulates tau alternative splicing. *Nucleic Acids Res.*, **40**, 9836–9849.
59. Vorlova, S., Rocco, G., Lefave, C.V., Jodelka, F.M., Hess, K., Hastings, M.L., Henke, E. and Cartegni, L. (2011) Induction of antagonistic soluble decoy receptor tyrosine kinases by intronic polyA activation. *Mol. Cell*, **43**, 927–939.
60. Dominski, Z. and Kole, R. (1993) Restoration of correct splicing in thalassemic pre-mRNA by antisense oligonucleotides. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 8673–8677.
61. Benchaoui, R., Robin, V. and Goyenvall, A. (2015) Gene and splicing therapies for neuromuscular diseases. *Front. Biosci.*, **20**, 1190–1233.
62. Siva, K., Covello, G. and Denti, M.A. (2014) Exon-skipping antisense oligonucleotides to correct missplicing in neurogenetic diseases. *Nucleic Acid Ther.*, **24**, 69–86.
63. McClorey, G. and Wood, M.J. (2015) An overview of the clinical application of antisense oligonucleotides for RNA-targeting therapies. *Curr. Opin. Pharmacol.*, **24**, 52–58.
64. Beaudet, A.L. and Meng, L. (2015) Gene-targeting pharmaceuticals for single-gene disorders. *Hum. Mol. Genet.*, **25**, R18–R26.
65. Disterer, P., Kryczka, A., Liu, Y., Badi, Y.E., Wong, J.J., Owen, J.S. and Khoo, B. (2014) Development of therapeutic splice-switching oligonucleotides. *Hum. Gene Ther.*, **25**, 587–598.
66. Arechavala-Gomez, V., Khoo, B. and Aartsma-Rus, A. (2014) Splicing modulation therapy in the treatment of genetic diseases. *Appl. Clin. Genet.*, **7**, 245–252.
67. Jirka, S. and Aartsma-Rus, A. (2015) An update on RNA-targeting therapies for neuromuscular disorders. *Curr. Opin. Neurol.*, **28**, 515–521.
68. Ellis, J.A., Vroom, E. and Muntoni, F. (2013) 195th ENMC International Workshop: Newborn screening for Duchenne muscular dystrophy 14–16th December, 2012, Naarden, The Netherlands. *Neuromuscul. Disord.*, **23**, 682–689.
69. Romitti, P.A., Zhu, Y., Puzhankara, S., James, K.A., Nabukera, S.K., Zamba, G.K., Ciafaloni, E., Cunniff, C., Druschel, C.M., Mathews, K.D. *et al.* (2015) Prevalence of Duchenne and Becker muscular dystrophies in the United States. *Pediatrics*, **135**, 513–521.
70. Mendell, J.R. and Lloyd-Puryear, M. (2013) Report of MDA muscle disease symposium on newborn screening for Duchenne muscular dystrophy. *Muscle Nerve*, **48**, 21–26.
71. Nowak, K.J. and Davies, K.E. (2004) Duchenne muscular dystrophy and dystrophin: Pathogenesis and opportunities for treatment. *EMBO Rep.*, **5**, 872–876.
72. Cox, G.F. and Kunkel, L.M. (1997) Dystrophies and heart disease. *Curr. Opin. Cardiol.*, **12**, 329–343.
73. Monaco, A.P., Bertelson, C.J., Liechti-Gallati, S., Moser, H. and Kunkel, L.M. (1988) An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. *Genomics*, **2**, 90–95.
74. Koenig, M., Beggs, A.H., Moyer, M., Scherpf, S., Heindrich, K., Bettecken, T., Meng, G., Muller, C.R., Lindlof, M., Kaariainen, H. *et al.* (1989) The molecular basis for Duchenne versus Becker muscular dystrophy: correlation of severity with type of deletion. *Am. J. Hum. Genet.*, **45**, 498–506.
75. Aartsma-Rus, A., Janson, A.A., Kaman, W.E., Bremmer-Bout, M., van Ommen, G.J., den Dunnen, J.T. and van Deutekom, J.C. (2004) Antisense-induced multiexon skipping for Duchenne muscular dystrophy makes more sense. *Am. J. Hum. Genet.*, **74**, 83–92.
76. Echigoya, Y. and Yokota, T. (2014) Skipping multiple exons of dystrophin transcripts using cocktail antisense oligonucleotides. *Nucleic Acid Ther.*, **24**, 57–68.
77. Takeshima, Y., Nishio, H., Sakamoto, H., Nakamura, H. and Matsuo, M. (1995) Modulation of in vitro splicing of the upstream

- intron by modifying an intra-exon sequence which is deleted from the dystrophin gene in dystrophin Kobe. *J. Clin. Invest.*, **95**, 515–520.
78. Duncley, M.G., Manoharan, M., Villiet, P., Eperon, I.C. and Dickson, G. (1998) Modification of splicing in the dystrophin gene in cultured Mdx muscle cells by antisense oligonucleotides. *Hum. Mol. Genet.*, **7**, 1083–1090.
  79. van Deutekom, J.C., Bremmer-Bout, M., Janson, A.A., Ginjaar, I.B., Baas, F., den Dunnen, J.T. and van Ommen, G.J. (2001) Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells. *Hum. Mol. Genet.*, **10**, 1547–1554.
  80. Mann, C.J., Honeyman, K., Cheng, A.J., Ly, T., Lloyd, F., Fletcher, S., Morgan, J.E., Partridge, T.A. and Wilton, S.D. (2001) Antisense-induced exon skipping and synthesis of dystrophin in the mdx mouse. *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 42–47.
  81. McClorey, G., Fletcher, S. and Wilton, S. (2005) Splicing intervention for Duchenne muscular dystrophy. *Curr. Opin. Pharmacol.*, **5**, 529–534.
  82. Heemskerk, H.A., de Winter, C.L., de Kimpe, S.J., van Kuik-Romeijn, P., Heuvelmans, N., Platenburg, G.J., van Ommen, G.J., van Deutekom, J.C. and Aartsma-Rus, A. (2009) In vivo comparison of 2'-O-methyl phosphorothioate and morpholino antisense oligonucleotides for Duchenne muscular dystrophy exon skipping. *J. Gene Med.*, **11**, 257–266.
  83. Alter, J., Lou, F., Rabinowitz, A., Yin, H., Rosenfeld, J., Wilton, S.D., Partridge, T.A. and Lu, Q.L. (2006) Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. *Nat. Med.*, **12**, 175–177.
  84. Tanganyika-de Winter, C.L., Heemskerk, H., Karnaoukh, T.G., van Putten, M., de Kimpe, S.J., van Deutekom, J. and Aartsma-Rus, A. (2012) Long-term Exon Skipping Studies With 2'-O-Methyl Phosphorothioate Antisense Oligonucleotides in Dystrophic Mouse Models. *Mol. Ther. Nucleic Acids*, **1**, e44.
  85. Aoki, Y., Yokota, T., Nagata, T., Nakamura, A., Tanihata, J., Saito, T., Duguez, S.M., Nagaraju, K., Hoffman, E.P., Partridge, T. et al. (2012) Bodywide skipping of exons 45-55 in dystrophic mdx52 mice by systemic antisense delivery. *Proc. Natl. Acad. Sci. U.S.A.*, **109**, 13763–13768.
  86. Takeshima, Y., Yagi, M., Wada, H., Ishibashi, K., Nishiyama, A., Kakumoto, M., Sakaeda, T., Saura, R., Okumura, K. and Matsuo, M. (2006) Intravenous infusion of an antisense oligonucleotide results in exon skipping in muscle dystrophin mRNA of Duchenne muscular dystrophy. *Pediatr. Res.*, **59**, 690–694.
  87. Koo, T. and Wood, M.J. (2013) Clinical trials using antisense oligonucleotides in duchenne muscular dystrophy. *Hum. Gene Ther.*, **24**, 479–488.
  88. (2015) A phase I/IIa clinical trial in duchenne muscular dystrophy using systemically delivered morpholino antisense oligomer to Skip Exon 53 (SKIP-NMD). *Hum. Gene Ther. Clin. Dev.*, **26**, 92–95.
  89. van Deutekom, J.C. and van Ommen, G.J. (2003) Advances in Duchenne muscular dystrophy gene therapy. *Nat. Rev. Genet.*, **4**, 774–783.
  90. De Angelis, F.G., Sthandier, O., Berarducci, B., Toso, S., Galluzzi, G., Ricci, E., Cossu, G. and Bozzoni, I. (2002) Chimeric snRNA molecules carrying antisense sequences against the splice junctions of exon 51 of the dystrophin pre-mRNA induce exon skipping and restoration of a dystrophin synthesis in Delta 48-50 DMD cells. *Proc. Natl. Acad. Sci. U.S.A.*, **99**, 9456–9461.
  91. Arechavala-Gomez, V., Graham, I.R., Popplewell, L.J., Adams, A.M., Aartsma-Rus, A., Kinali, M., Morgan, J.E., van Deutekom, J.C., Wilton, S.D., Dickson, G. et al. (2007) Comparative analysis of antisense oligonucleotide sequences for targeted skipping of exon 51 during dystrophin pre-mRNA splicing in human muscle. *Hum. Gene Ther.*, **18**, 798–810.
  92. Sazani, P., Weller, D.L. and Shrewsbury, S.B. (2010) Safety pharmacology and genotoxicity evaluation of AVI-4658. *Int. J. Toxicol.*, **29**, 143–156.
  93. Sazani, P., Ness, K.P., Weller, D.L., Poage, D., Nelson, K. and Shrewsbury, A.S. (2011) Chemical and mechanistic toxicology evaluation of exon skipping phosphorodiamidate morpholino oligomers in mdx mice. *Int. J. Toxicol.*, **30**, 322–333.
  94. Sazani, P., Ness, K.P., Weller, D.L., Poage, D.W., Palyada, K. and Shrewsbury, S.B. (2011) Repeat-dose toxicology evaluation in cynomolgus monkeys of AVI-4658, a phosphorodiamidate morpholino oligomer (PMO) drug for the treatment of duchenne muscular dystrophy. *Int. J. Toxicol.*, **30**, 313–321.
  95. Kinali, M., Arechavala-Gomez, V., Feng, L., Cirak, S., Hunt, D., Adkin, C., Guglieri, M., Ashton, E., Abbs, S., Nihoyannopoulos, P. et al. (2009) Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study. *Lancet Neurol.*, **8**, 918–928.
  96. Mendell, J.R., Rodino-Klapac, L.R., Sahenk, Z., Roush, K., Bird, L., Lowes, L.P., Alfano, L., Gomez, A.M., Lewis, S., Kota, J. et al. (2013) Eteplirsen for the treatment of Duchenne muscular dystrophy. *Ann. Neurol.*, **74**, 637–647.
  97. van Deutekom, J.C., Janson, A.A., Ginjaar, I.B., Frankhuizen, W.S., Aartsma-Rus, A., Bremmer-Bout, M., den Dunnen, J.T., Koop, K., van der Kooi, A.J., Goemans, N.M. et al. (2007) Local dystrophin restoration with antisense oligonucleotide PRO051. *New Engl. J. Med.*, **357**, 2677–2686.
  98. Voit, T., Topaloglu, H., Straub, V., Muntoni, F., Deconinck, N., Campion, G., De Kimpe, S.J., Eagle, M., Guglieri, M., Hood, S. et al. (2014) Safety and efficacy of drisapersen for the treatment of Duchenne muscular dystrophy (DEMAND II): an exploratory, randomised, placebo-controlled phase 2 study. *Lancet Neurol.*, **13**, 987–996.
  99. Goemans, N.M., Tulinus, M., van den Akker, J.T., Burm, B.E., Ekhardt, P.F., Heuvelmans, N., Holling, T., Janson, A.A., Platenburg, G.J., Sipkens, J.A. et al. (2011) Systemic administration of PRO051 in Duchenne's muscular dystrophy. *New Engl. J. Med.*, **364**, 1513–1522.
  100. Lu, Q.L., Cirak, S. and Partridge, T. (2014) What can we learn from clinical trials of exon skipping for DMD? *Mol. Ther. Nucleic Acids*, **3**, e152.
  101. Arechavala-Gomez, V., Anthony, K., Morgan, J. and Muntoni, F. (2012) Antisense oligonucleotide-mediated exon skipping for Duchenne muscular dystrophy: progress and challenges. *Curr. Gene Ther.*, **12**, 152–160.
  102. Ricotti, V., Muntoni, F. and Voit, T. (2015) Challenges of clinical trial design for DMD. *Neuromuscul. Disord.*, **25**, 932–935.
  103. Prele, A., Chianese, L., Moggio, M., Gallanti, A., Sciacco, M., Checcarelli, N., Comi, G., Scarpini, E., Bonilla, E. and Scarlato, G. (1991) Appearance and localization of dystrophin in normal human fetal muscle. *Int. J. Dev. Neurosci.*, **9**, 607–612.
  104. Wu, B., Cloer, C., Lu, P., Milazi, S., Shaban, M., Shah, S.N., Marston-Poe, L., Moulton, H.M. and Lu, Q.L. (2014) Exon skipping restores dystrophin expression, but fails to prevent disease progression in later stage dystrophic dko mice. *Gene Ther.*, **21**, 785–793.
  105. Merrick, D., Stadler, L.K., Lerner, D. and Smith, J. (2009) Muscular dystrophy begins early in embryonic development deriving from stem cell loss and disrupted skeletal muscle formation. *Dis. Models Mech.*, **2**, 374–388.
  106. Kolb, S.J. and Kissel, J.T. (2015) Spinal muscular atrophy. *Neurol. Clin.*, **33**, 831–846.
  107. Prior, T.W., Snyder, P.J., Rink, B.D., Pearl, D.K., Pyatt, R.E., Mihal, D.C., Conlan, T., Schmalz, B., Montgomery, L., Ziegler, K. et al. (2010) Newborn and carrier screening for spinal muscular atrophy. *Am. J. Med. Genet. A*, **152**, 1608–1616.
  108. Sugarman, E.A., Nagan, N., Zhu, H., Akmaev, V.R., Zhou, Z., Rohlf, E.M., Flynn, K., Hendrickson, B.C., Scholl, T., Sirko-Osada, D.A. et al. (2012) Pan-ethnic carrier screening and prenatal diagnosis for spinal muscular atrophy: clinical laboratory analysis of >72,400 specimens. *Eur. J. Hum. Genet.*, **20**, 27–32.
  109. Larson, J.L., Silver, A.J., Chan, D., Borroto, C., Spurrier, B. and Silver, L.M. (2015) Validation of a high resolution NGS method for detecting spinal muscular atrophy carriers among phase 3 participants in the 1000 Genomes Project. *BMC Med. Genet.*, **16**, 100–113.
  110. Finkel, R.S., McDermott, M.P., Kaufmann, P., Darras, B.T., Chung, W.K., Sproule, D.M., Kang, P.B., Foley, A.R., Yang, M.L., Martens, W.B. et al. (2014) Observational study of spinal muscular atrophy type I and implications for clinical trials. *Neurology*, **83**, 810–817.
  111. Farrar, M.A., Vucic, S., Johnston, H.M., du Sart, D. and Kiernan, M.C. (2013) Pathophysiological insights derived by natural

- history and motor function of spinal muscular atrophy. *J. Pediatr.*, **162**, 155–159.
112. Rudnik-Schoneborn, S., Berg, C., Zerres, K., Betzler, C., Grimm, T., Eggermann, T., Eggermann, K., Wirth, R., Wirth, B. and Heller, R. (2009) Genotype-phenotype studies in infantile spinal muscular atrophy (SMA) type I in Germany: implications for clinical trials and genetic counselling. *Clin. Genet.*, **76**, 168–178.
  113. Lorson, C.L., Hahnen, E., Androphy, E.J. and Wirth, B. (1999) A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. *Proc. Natl. Acad. Sci. U.S.A.*, **96**, 6307–6311.
  114. Monani, U.R., Lorson, C.L., Parsons, D.W., Prior, T.W., Androphy, E.J., Burghes, A.H. and McPherson, J.D. (1999) A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2. *Hum. Mol. Genet.*, **8**, 1177–1183.
  115. Cartegni, L. and Krainer, A.R. (2002) Disruption of an SF2/ASF-dependent exonic splicing enhancer in SMN2 causes spinal muscular atrophy in the absence of SMN1. *Nat. Genet.*, **30**, 377–384.
  116. Cartegni, L., Hastings, M.L., Calarco, J.A., de Stanchina, E. and Krainer, A.R. (2006) Determinants of exon 7 splicing in the spinal muscular atrophy genes, SMN1 and SMN2. *Am. J. Hum. Genet.*, **78**, 63–77.
  117. Kashima, T. and Manley, J.L. (2003) A negative element in SMN2 exon 7 inhibits splicing in spinal muscular atrophy. *Nat. Genet.*, **34**, 460–463.
  118. Cho, S. and Dreyfuss, G. (2010) A degron created by SMN2 exon 7 skipping is a principal contributor to spinal muscular atrophy severity. *Genes Dev.*, **24**, 438–442.
  119. Lorson, C.L. and Androphy, E.J. (2000) An exonic enhancer is required for inclusion of an essential exon in the SMA-determining gene SMN. *Hum. Mol. Genet.*, **9**, 259–265.
  120. Lefebvre, S., Burglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L., Benichou, B., Cruaud, C., Millasseau, P., Zeviani, M. et al. (1995) Identification and characterization of a spinal muscular atrophy-determining gene. *Cell*, **80**, 155–165.
  121. Gavrilov, D.K., Shi, X., Das, K., Gilliam, T.C. and Wang, C.H. (1998) Differential SMN2 expression associated with SMA severity. *Nat. Genet.*, **20**, 230–231.
  122. Lefebvre, S., Burlet, P., Liu, Q., Bertrand, S., Clermont, O., Munnich, A., Dreyfuss, G. and Melki, J. (1997) Correlation between severity and SMN protein level in spinal muscular atrophy. *Nat. Genet.*, **16**, 265–269.
  123. McAndrew, P.E., Parsons, D.W., Simard, L.R., Rochette, C., Ray, P.N., Mendell, J.R., Prior, T.W. and Burghes, A.H. (1997) Identification of proximal spinal muscular atrophy carriers and patients by analysis of SMNT and SMNC gene copy number. *Am. J. Hum. Genet.*, **60**, 1411–1422.
  124. Wirth, B., Brichta, L., Schrank, B., Lochmuller, H., Blick, S., Baasner, A. and Heller, R. (2006) Mildly affected patients with spinal muscular atrophy are partially protected by an increased SMN2 copy number. *Hum. Genet.*, **119**, 422–428.
  125. Mercuri, E., Bertini, E. and Iannaccone, S.T. (2012) Childhood spinal muscular atrophy: controversies and challenges. *Lancet Neurol.*, **11**, 443–452.
  126. Lim, S.R. and Hertel, K.J. (2001) Modulation of survival motor neuron pre-mRNA splicing by inhibition of alternative 3' splice site pairing. *J. Biol. Chem.*, **276**, 45476–45483.
  127. Miyajima, H., Miyaso, H., Okumura, M., Kurisu, J. and Imaizumi, K. (2002) Identification of a cis-acting element for the regulation of SMN exon 7 splicing. *J. Biol. Chem.*, **277**, 23271–23277.
  128. Cartegni, L. and Krainer, A.R. (2003) Correction of disease-associated exon skipping by synthetic exon-specific activators. *Nat. Struct. Biol.*, **10**, 120–125.
  129. Skordis, L.A., Dunckley, M.G., Yue, B., Eperon, I.C. and Muntoni, F. (2003) Bifunctional antisense oligonucleotides provide a trans-acting splicing enhancer that stimulates SMN2 gene expression in patient fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.*, **100**, 4114–4119.
  130. Tisdale, S. and Pellizzoni, L. (2015) Disease mechanisms and therapeutic approaches in spinal muscular atrophy. *J. Neurosci.*, **35**, 8691–8700.
  131. Mitrpant, C., Porensky, P., Zhou, H., Price, L., Muntoni, F., Fletcher, S., Wilton, S.D. and Burghes, A.H. (2013) Improved antisense oligonucleotide design to suppress aberrant SMN2 gene transcript processing: towards a treatment for spinal muscular atrophy. *PLoS One*, **8**, e62114.
  132. Zhou, H., Janghra, N., Mitrpant, C., Dickinson, R.L., Anthony, K., Price, L., Eperon, I.C., Wilton, S.D., Morgan, J. and Muntoni, F. (2013) A novel morpholino oligomer targeting ISS-N1 improves rescue of severe spinal muscular atrophy transgenic mice. *Hum. Gene Ther.*, **24**, 331–342.
  133. Osman, E.Y., Miller, M.R., Robbins, K.L., Lombardi, A.M., Atkinson, A.K., Brehm, A.J. and Lorson, C.L. (2014) Morpholino antisense oligonucleotides targeting intronic repressor Element1 improve phenotype in SMA mouse models. *Hum. Mol. Genet.*, **23**, 4832–4845.
  134. Keil, J.M., Seo, J., Howell, M.D., Hsu, W.H., Singh, R.N. and DiDonato, C.J. (2014) A short antisense oligonucleotide ameliorates symptoms of severe mouse models of spinal muscular atrophy. *Mol. Ther. Nucleic Acids*, **3**, e174.
  135. Singh, N.N., Shishimorova, M., Cao, L.C., Gangwani, L. and Singh, R.N. (2009) A short antisense oligonucleotide masking a unique intronic motif prevents skipping of a critical exon in spinal muscular atrophy. *RNA Biol.*, **6**, 341–350.
  136. Singh, N.K., Singh, N.N., Androphy, E.J. and Singh, R.N. (2006) Splicing of a critical exon of human Survival Motor Neuron is regulated by a unique silencer element located in the last intron. *Mol. Cell. Biol.*, **26**, 1333–1346.
  137. Hua, Y., Vickers, T.A., Okunola, H.L., Bennett, C.F. and Krainer, A.R. (2008) Antisense masking of an hnRNP A1/A2 intronic splicing silencer corrects SMN2 splicing in transgenic mice. *Am. J. Hum. Genet.*, **82**, 834–848.
  138. Porensky, P.N., Mitrpant, C., McGovern, V.L., Bevan, A.K., Foust, K.D., Kaspar, B.K., Wilton, S.D. and Burghes, A.H. (2012) A single administration of morpholino antisense oligomer rescues spinal muscular atrophy in mouse. *Hum. Mol. Genet.*, **21**, 1625–1638.
  139. Passini, M.A., Bu, J., Richards, A.M., Kinnecom, C., Sardi, S.P., Stanek, L.M., Hua, Y., Rigo, F., Matsun, J., Hung, G. et al. (2011) Antisense oligonucleotides delivered to the mouse CNS ameliorate symptoms of severe spinal muscular atrophy. *Sci. Transl. Med.*, **3**, 72ra18.
  140. Sahashi, K., Ling, K.K., Hua, Y., Wilkinson, J.E., Nomakuchi, T., Rigo, F., Hung, G., Xu, D., Jiang, Y.P., Lin, R.Z. et al. (2013) Pathological impact of SMN2 mis-splicing in adult SMA mice. *EMBO Mol. Med.*, **5**, 1586–1601.
  141. Hua, Y., Liu, Y.H., Sahashi, K., Rigo, F., Bennett, C.F. and Krainer, A.R. (2015) Motor neuron cell-nonautonomous rescue of spinal muscular atrophy phenotypes in mild and severe transgenic mouse models. *Genes Dev.*, **29**, 288–297.
  142. Chiriboga, C.A., Swoboda, K.J., Darras, B.T., Iannaccone, S.T., Montes, J., De Vivo, D.C., Norris, D.A., Bennett, C.F. and Bishop, K.M. (2016) Results from a phase 1 study of nusinersen (ISIS-SMNRx) in children with spinal muscular atrophy. *Neurology*, **86**, 890–897.
  143. Verhaart, I.E., Tanganyika-de Winter, C.L., Karnaoukh, T.G., Kolfschoten, I.G., de Kimpe, S.J., van Deutekom, J.C. and Aartsma-Rus, A. (2013) Dose-dependent pharmacokinetic profiles of 2'-O-methyl phosphorothioate antisense oligonucleotides in mdx mice. *Nucleic Acid Ther.*, **23**, 228–237.
  144. Svasti, S., Suwanmanee, T., Fucharoen, S., Moulton, H.M., Nelson, M.H., Maeda, N., Smithies, O. and Kole, R. (2009) RNA repair restores hemoglobin expression in IVS2-654 thalassemic mice. *Proc. Natl. Acad. Sci. U.S.A.*, **106**, 1205–1210.
  145. Taniguchi-Ikeda, M., Kobayashi, K., Kanagawa, M., Yu, C.C., Mori, K., Oda, T., Kuga, A., Kurahashi, H., Akman, H.O., DiMauro, S. et al. (2011) Pathogenic exon-trapping by SVA retrotransposon and rescue in Fukuyama muscular dystrophy. *Nature*, **478**, 127–131.
  146. Osorio, F.G., Navarro, C.L., Cadinanos, J., Lopez-Mejia, I.C., Quiros, P.M., Bartoli, C., Rivera, J., Tazi, J., Guzman, G., Varela, I. et al. (2011) Splicing-directed therapy in a new mouse model of human accelerated aging. *Sci. Transl. Med.*, **3**, 106ra107.
  147. Lee, J.M., Nobumori, C., Tu, Y., Choi, C., Yang, S.H., Jung, H.J., Vickers, T.A., Rigo, F., Bennett, C.F., Young, S.G. et al. (2016) Modulation of LMNA splicing as a strategy to treat prelamin A diseases. *J. Clin. Invest.*, **126**, 1592–1602.

148. Wheeler, T.M., Lueck, J.D., Swanson, M.S., Dirksen, R.T. and Thornton, C.A. (2007) Correction of CIC-1 splicing eliminates chloride channelopathy and myotonia in mouse models of myotonic dystrophy. *J. Clin. Invest.*, **117**, 3952–3957.
149. Bestas, B., Moreno, P.M., Blomberg, K.E., Mohammad, D.K., Saleh, A.F., Sutlu, T., Nordin, J.Z., Guterstam, P., Gustafsson, M.O., Kharazi, S. *et al.* (2014) Splice-correcting oligonucleotides restore BTK function in X-linked agammaglobulinemia model. *J. Clin. Invest.*, **124**, 4067–4081.
150. Mourich, D.V., Oda, S.K., Schnell, F.J., Crumley, S.L., Hauck, L.L., Moentenich, C.A., Marshall, N.B., Hinrichs, D.J. and Iversen, P.L. (2014) Alternative splice forms of CTLA-4 induced by antisense mediated splice-switching influences autoimmune diabetes susceptibility in NOD mice. *Nucleic Acid Ther.*, **24**, 114–126.
151. Bauman, J.A., Li, S.D., Yang, A., Huang, L. and Kole, R. (2010) Anti-tumor activity of splice-switching oligonucleotides. *Nucleic Acids Res.*, **38**, 8348–8356.
152. Wielsen, T.O., Sorensen, S., Dagnaes-Hansen, F., Kjems, J. and Sorensen, B.S. (2013) Directing HER4 mRNA expression towards the CYT2 isoform by antisense oligonucleotide decreases growth of breast cancer cells in vitro and in vivo. *Br. J. Cancer*, **108**, 2291–2298.
153. Dewaele, M., Tabaglio, T., Willekens, K., Bezzi, M., Teo, S.X., Low, D.H., Koh, C.M., Rambow, F., Fiers, M., Rogiers, A. *et al.* (2016) Antisense oligonucleotide-mediated MDM4 exon 6 skipping impairs tumor growth. *J. Clin. Invest.*, **126**, 68–84.
154. Zammarchi, F., de Stanchina, E., Bournazou, E., Supakorndej, T., Martires, K., Riedel, E., Corben, A.D., Bromberg, J.F. and Cartegni, L. (2011) Antitumorigenic potential of STAT3 alternative splicing modulation. *Proc. Natl. Acad. Sci. U.S.A.*, **108**, 17779–17784.
155. Yilmaz-Elis, A.S., Aartsma-Rus, A., t Hoen, P.A., Safdar, H., Breukel, C., van Vlijmen, B.J., van Deutekom, J., de Kimpe, S., van Ommen, G.J. and Verbeek, J.S. (2013) Inhibition of IL-1 signaling by antisense oligonucleotide-mediated exon skipping of IL-1 Receptor Accessory Protein (IL-1RAcP). *Mol. Ther. Nucleic Acids*, **2**, e66.
156. Graziewicz, M.A., Tarrant, T.K., Buckley, B., Roberts, J., Fulton, L., Hansen, H., Orum, H., Kole, R. and Sazani, P. (2008) An endogenous TNF- $\alpha$  antagonist induced by splice-switching oligonucleotides reduces inflammation in hepatitis and arthritis mouse models. *Mol. Ther.*, **16**, 1316–1322.
157. Owen, L.A., Uehara, H., Cahoon, J., Huang, W., Simonis, J. and Ambati, B.K. (2012) Morpholino-mediated increase in soluble Flt-1 expression results in decreased ocular and tumor neovascularization. *PLoS One*, **7**, e33576.
158. Uehara, H., Cho, Y., Simonis, J., Cahoon, J., Archer, B., Luo, L., Das, S.K., Singh, N., Ambati, J. and Ambati, B.K. (2013) Dual suppression of hemangiogenesis and lymphangiogenesis by splice-shifting morpholinos targeting vascular endothelial growth factor receptor 2 (KDR). *FASEB J.*, **27**, 76–85.
159. Gedicke-Hornung, C., Behrens-Gawlik, V., Reischmann, S., Geertz, B., Stimpel, D., Weinberger, F., Schlossarek, S., Precigout, G., Braren, I., Eschenhagen, T. *et al.* (2013) Rescue of cardiomyopathy through U7snRNA-mediated exon skipping in Mybpc3-targeted knock-in mice. *EMBO Mol. Med.*, **5**, 1060–1077.
160. Gramlich, M., Pane, L.S., Zhou, Q., Chen, Z., Murgia, M., Schotterl, S., Goedel, A., Metzger, K., Brade, T., Parrotta, E. *et al.* (2015) Antisense-mediated exon skipping: a therapeutic strategy for titin-based dilated cardiomyopathy. *EMBO Mol. Med.*, **7**, 562–576.
161. Salewski, B., Hildebrand, G., Rothe, S., Parplys, A.C., Radszewski, J., Kieslich, M., Wessendorf, P., Krenzlin, H., Borgmann, K., Nussenzweig, A. *et al.* (2016) Directed alternative splicing in Nijmegen Breakage Syndrome: Proof of principle concerning its therapeutical application. *Mol. Ther.*, **24**, 117–124.
162. Panchal, R.G., Mourich, D.V., Bradfute, S., Hauck, L.L., Warfield, K.L., Iversen, P.L. and Bavari, S. (2014) Induced IL-10 splice altering approach to antiviral drug discovery. *Nucleic Acid Ther.*, **24**, 179–185.
163. Evers, M.M., Tran, H.D., Zalachoras, I., Meijer, O.C., den Dunnen, J.T., van Ommen, G.J., Aartsma-Rus, A. and van Roon-Mom, W.M. (2014) Preventing formation of toxic N-terminal huntingtin fragments through antisense oligonucleotide-mediated protein modification. *Nucleic Acid Ther.*, **24**, 4–12.
164. Disterer, P., Al-Shawi, R., Ellmerich, S., Waddington, S.N., Owen, J.S., Simons, J.P. and Khoo, B. (2013) Exon skipping of hepatic APOB pre-mRNA with splice-switching oligonucleotides reduces LDL cholesterol in vivo. *Mol. Ther.*, **21**, 602–609.
165. Kang, J.K., Malerba, A., Popplewell, L., Foster, K. and Dickson, G. (2011) Antisense-induced myostatin exon skipping leads to muscle hypertrophy in mice following octa-guanidine morpholino oligomer treatment. *Mol. Ther.*, **19**, 159–164.
166. Lu-Nguyen, N.B., Jarmin, S.A., Saleh, A.F., Popplewell, L., Gait, M.J. and Dickson, G. (2015) Combination antisense treatment for destructive exon skipping of myostatin and open reading frame rescue of dystrophin in neonatal mdx mice. *Mol. Ther.*, **23**, 1341–1348.
167. Clayton, N.P., Nelson, C.A., Weeden, T., Taylor, K.M., Moreland, R.J., Scheule, R.K., Phillips, L., Leger, A.J., Cheng, S.H. and Wentworth, B.M. (2014) Antisense oligonucleotide-mediated suppression of Muscle Glycogen Synthase 1 synthesis as an approach for substrate reduction therapy of pompe disease. *Mol. Ther.*, **3**, e206.
168. Evers, M.M., Tran, H.D., Zalachoras, I., Pepers, B.A., Meijer, O.C., den Dunnen, J.T., van Ommen, G.J., Aartsma-Rus, A. and van Roon-Mom, W.M. (2013) Ataxin-3 protein modification as a treatment strategy for spinocerebellar ataxia type 3: removal of the CAG containing exon. *Neurobiol. Dis.*, **58**, 49–56.

# EXHIBIT 50

## Review

---

# Exon-Skipping in Duchenne Muscular Dystrophy

Shin'ichi Takeda<sup>a</sup>, Paula R. Clemens<sup>b</sup> and Eric P. Hoffman<sup>c,\*</sup>

<sup>a</sup>*Honorary Director General, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), Kodaira, Japan*

<sup>b</sup>*Professor and Vice Chair of VA Affairs, Department of Neurology, University of Pittsburgh School of Medicine, Division Chief, Neurology, Medical Service Line, VA Pittsburgh Healthcare System, Pittsburgh, PA USA*

<sup>c</sup>*Professor, Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, Binghamton University – State University of New York, Binghamton, NY USA*

Pre-press 24 June 2021

**Abstract.** Duchenne muscular dystrophy (DMD) is a devastating, rare disease. While clinically described in the 19<sup>th</sup> century, the genetic foundation of DMD was not discovered until more than 100 years later. This genetic understanding opened the door to the development of genetic treatments for DMD. Over the course of the last 30 years, the research that supports this development has moved into the realm of clinical trials and regulatory drug approvals. Exon skipping to therapeutically restore the frame of an out-of-frame dystrophin mutation has taken center stage in drug development for DMD. The research reviewed here focuses on the clinical development of exon skipping for the treatment of DMD. In addition to the generation of clinical treatments that are being used for patient care, this research sets the stage for future therapeutic development with a focus on increasing efficacy while providing safety and addressing the multi-systemic aspects of DMD.

## THE DMD GENE AND DYSTROPHIN PROTEIN STRUCTURE AND FUNCTION

Gene mutations in the 2.24 million base pair *DMD* gene on the X chromosome result in biochemical loss or abnormalities of the dystrophin protein. The *DMD* gene has multiple gene promoters driving expression of different mRNA (and encoded protein) isoforms (Fig. 1). The ‘full-length’ 14 kb mRNA using the 3 most proximal 5’ gene promoters (Dp427B, Dp427M, Dp427P) contains 79 exons and

encodes a 427 kDa membrane cytoskeletal protein (dystrophin), that is expressed in all skeletal muscles, smooth muscles (vascular and visceral), heart, peripheral nerve, and some neurons. The *DMD* gene also contains multiple downstream distal promoters encoding shorter mRNA and protein isoforms (Dp260, Dp140, Dp116), with the shortest Dp71 (Dystrophin protein, 71 kDa) showing relatively ubiquitous expression in non-muscle and nerve cells (Fig. 1) [1].

Dystrophin is a component of the intracellular membrane cytoskeleton where it interacts with actin filaments, intermediate filaments, and transmembrane proteins that themselves interact with the extracellular basal lamina. It has been dubbed a ‘broad membrane integrator’ [2]. The primary biochemical

---

\*Correspondence to: Eric P. Hoffman, Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, Binghamton University – State University of New York, Binghamton, NY, USA. E-mail: ericphoffman@gmail.com.

S344

S. Takeda et al. / Exon-Skipping in Duchenne Muscular Dystrophy

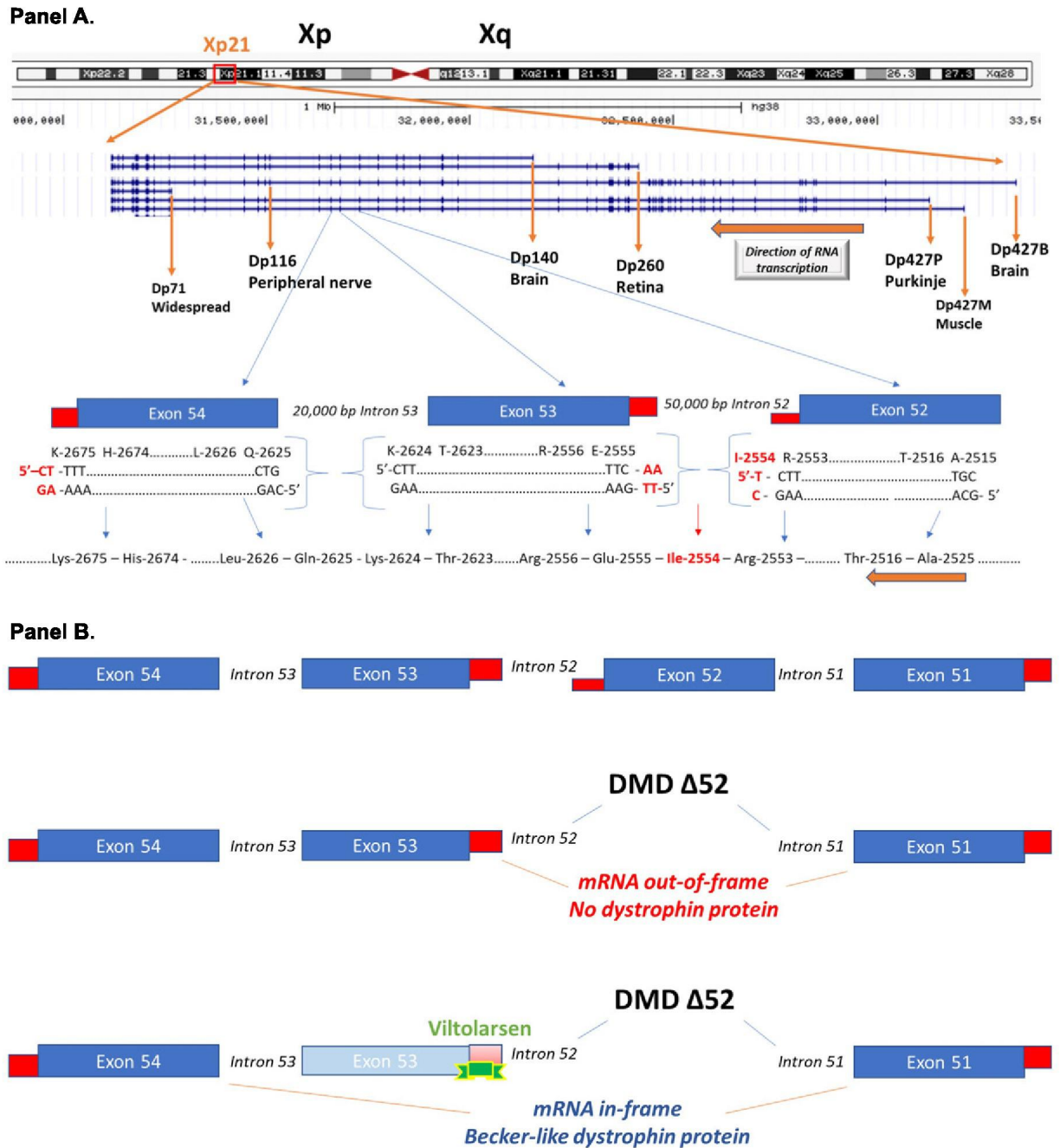


Fig. 1. Schematic of the *DMD* gene and exon skipping. **Panel A:** A schematic of the *DMD* gene from the Genome Browser (genome.ucsc.edu) at Xp21 with encoded mRNA transcripts is shown. Gene transcription is shown from left to right, with the three gene promoters driving expression of the full-length 427 kDa dystrophin (Dp427B, Dp427M, Dp427P), as well as down-stream gene promoters driving smaller molecular weight dystrophin proteins (Dp260, Dp140, Dp116, Dp71). Also shown is an expansion of exons 52, 53, and 54. The amino acids and encoding triplet codons are provided at the ends of each of these exons. Exon 52 ends in an incomplete codon for isoleucine (I-2554), requiring the last two bases from exon 53 to complete the codon. In contrast, exon 53 ends with a complete codon for lysine (K-2624), splicing to exon 54 that starts with a complete codon for glutamine (Q-2625). A gene mutation deleting exon 53 would then be out-of-frame, as an incomplete codon ending exon 52 would be fused to a complete codon on exon 54, leading to a frame shift in the resulting dystrophin mRNA. **Panel B:** This shows the consequence of drug-induced exon skipping by viltolarsen targeted to exon 53. A boy with DMD is shown as having a deletion mutation of exon 52, and when this patient's dystrophin mRNA splices together the remaining exons (exon 51 to exon 52), this leads to a frame shift, mRNA out-of-frame, and no dystrophin protein. Viltolarsen binds to exon 53, and blocks its inclusion in the dystrophin mRNA. The drug-induced splicing of exon 51 to exon 54 results in an in-frame dystrophin mRNA, and Becker-like dystrophin protein.

role of dystrophin in skeletal muscle myofibers is to increase the stability of the plasma membrane, protecting it from force-related membrane disruptions. In this way, its biochemical role is similar to the structurally-related spectrin protein in red blood cells, where spectrin similarly imparts deformability and stability of plasma membranes (of erythrocytes) as they pass through small capillaries. While dystrophin does not have signaling or enzymatic roles itself, it binds directly or indirectly to multiple other proteins that do have signaling or enzymatic roles, such as neuronal nitric oxide synthase (nNOS).

The *DMD* gene mutations that inactivate the gene or mRNA such that little or no dystrophin is produced (e.g. null mutations) lead to dystrophin deficiency. However, the location of an inactivating (frame-shift or nonsense) mutation within the *DMD* gene can differentially affect different isoforms. For example, a mutation within the first 29 exons of the gene would be expected to inactivate mRNA and protein from the full-length brain, muscle, and Purkinje cell gene promoters (Dp427B, Dp427M, Dp427P), but leave the downstream Dp260 (retina), Dp140 (brain), Dp116 (peripheral nerve) and Dp71 (widespread) dystrophin proteins intact [Fig. 1]. On the other hand, mutations in the last 17 exons (3' end of the gene) would be expected to lead to deficiency of all dystrophin isoforms in all tissues. Clinical findings are consistent with this, where loss of Dp260 (retina) leads to loss of night vision and distinctive changes in electroretinography findings, and this phenotype correlates with the location of the *DMD* gene mutation and predicted effect on the Dp260 isoform. The retinopathy phenotype maps to distal mutations downstream of Dp260 (retina) isoform [3], but there appears to be retinal sensitivity to ischemia that maps to the full length Dp427 isoform [4]. Likewise, mutations in the 3' end of the gene, removing more dystrophin isoforms, are correlated with more severe cognitive involvement and developmental brain abnormalities [5–7].

While the diagnostic term of a 'muscular dystrophy' is often thought of as a disorder restricted to skeletal muscle structure and function, increasing knowledge of DMD suggests that the clinical disorder has features of a multi-system disease (syndrome) with functional defects of vascular smooth muscle, visceral smooth muscle, heart, and brain/nerve. In the majority of cases, the skeletal muscle disease predominates, but abnormalities of other tissues contribute to the overall clinical picture. Use of assisted ventilation extends patient lifespan; most ventilated patients succumb to cardiac disease. This is a point to

consider when developing and evaluating therapeutic approaches to DMD.

## BECKER MUSCULAR DYSTROPHY AND CLINICAL VARIABILITY

The diagnosis of Becker muscular dystrophy (BMD) was originally reserved for male patients from X-linked recessive families segregating a muscular dystrophy that was clinically milder than DMD. With the cloning of the *DMD* gene, identification of dystrophin, and advent of molecular diagnostics, the diagnosis of BMD became synonymous with present but abnormal dystrophin protein in skeletal muscle biopsies [8, 9]. It soon became apparent in practice that a genetic characteristic of most cases of BMD, an in-frame deletion in the dystrophin gene, was not always concordant with a milder 'Becker-like' phenotype. This discordance impacts the design of human clinical trials focused on BMD therapeutics.

While all BMD patients show present but biochemically abnormal dystrophin in muscle, the gene mutations causing the abnormal dystrophin are highly variable, and the precise biochemical perturbations of the dystrophin protein similarly highly variable. The most common gene mutations in BMD are two in-frame deletions ( $\Delta 45-47$  [30%];  $\Delta 45-48$  [20%]) [10]. The 'reading frame rule' defined by the out-of-frame (inactivating) *DMD* gene mutations in the severe DMD, and the in-frame (residual function) *DMD* gene mutations in BMD is correlated with protein findings in about 75% of cases; there are many exceptions to this rule. The exceptions follow some patterns. Gene mutations in the 5' (beginning) of the gene can be out-of-frame (DMD) but show dystrophin protein on muscle biopsy and a milder clinical picture, both features that are consistent with the diagnosis of BMD. This is often due to use of alternative AUG initiator codons in mRNAs (downstream of the authentic AUG), and escape of nonsense mediated decay (cellular degradation of out-of-frame mRNAs). Out-of-frame exon 44 or 45 skippable mutations can show low levels of residual dystrophin due to endogenous (naturally occurring) alternative splicing creating low levels of in-frame transcripts, with about half of patients showing a milder phenotype [11]. Splice site mutations are 'leaky' in that they are often non-null (some normal dystrophin) [12]. Efforts to predict the clinical outcomes of different in-frame deletions based on systematic analyses of

large DNA/phenotype databases find considerable heterogeneity in observed phenotypes [13].

Very low levels of normal dystrophin can be associated with a clinical picture that can be milder than typical DMD [15]. That said, low levels of both normal and Becker-like dystrophins can also be associated with a typical DMD phenotype. Other factors over and above gene mutation type and dystrophin protein content of muscle contribute to variation in clinical phenotypes of both DMD and BMD, including genetic modifiers [14] and socioeconomic status [15]. Natural history studies of BMD patient cohorts have shown marked clinical variability, even between those with the same in-frame mutation, ranging from just slightly milder than DMD to asymptomatic [13, 16, 17]. Importantly, muscle MRI findings of the degree of fatty replacement of skeletal muscles appear more correlated with clinical symptoms than either dystrophin protein content or gene mutation [18, 20]. The fact that MRI imaging of fatty replacement is so well-correlated with patient functional ability likely reflects the importance of the variable inflammation and fibrosis pathways downstream of the primary gene and protein defect, and the variability of different muscle groups in terms of moving into the fatty replacement phase associated with functional disability.

Overall, the expectations of therapies directed towards low levels of dystrophin should acknowledge that all approaches aim to rescue or deliver dystrophin protein that is biochemically abnormal, and, therefore, partially functional (exon-skipping, gene therapy, CRISPR DNA editing). Success at achieving any level (low or high) of partially function dystrophin protein will almost certainly be associated with marked clinical variability from patient to patient, and within a patient from muscle to muscle.

### **THERAPEUTIC APPROACH OF CONVERTING DUCHENNE TO BECKER MUSCULAR DYSTROPHY**

Therapeutic approaches that aim to restore partially functional muscle dystrophin in patients with DMD focus on one of three approaches: 1. gene delivery using viral vectors; 2. stop codon read-through; 3. converting out-of-frame mutations to in-frame mutations (exon skipping; multiple approaches).

For gene delivery using viral vectors, the limited carrying capacity of the most suitable viral vectors based on the adeno-associated virus (AAV) requires

use of a highly modified smaller molecular weight versions of dystrophin called micro-dystrophins (~150 kDa compared to normal 427 kDa dystrophin). The micro-dystrophins are semi-functional proteins that have removed over half the normal dystrophin amino acid sequence. The biochemically abnormal dystrophin delivered with gene therapy is, in part, similar to 'Becker-like' dystrophins that occur naturally in patients with BMD or are induced by exon-skipping as a treatment for patients with DMD. Of note, the AAV-driven dystrophins are much smaller than those seen in Becker muscular dystrophy patients. Pre-clinical data in the *mdx* mouse [19–21] and CXMD dog [22–24] demonstrated functional benefit of AAV-delivered *de novo* micro-dystrophin. Preliminary results from a human clinical trial of AAV gene therapy have shown high level expression of micro-dystrophin in DMD patient muscle [25], with improvements in MRI fat fraction findings through 24 months post-treatment [26]. A key question with gene therapy in DMD is persistence of effect, as re-delivery is anticipated to be significantly limited by antibody responses against the second administration of the viral delivery vehicle, and degeneration/regeneration cycles of DMD muscle. Also, many DMD boys have pre-existing immunity to certain serotypes of AAV that are used for gene delivery, and this currently excludes these patients from receiving gene therapy [27]. Immunosuppressant strategies to address this limitation of gene therapy are being developed [28].

For stop codon read-through, about 10–15% of boys with DMD have an amino-acid codon mutated to a premature stop codon, and enabling the ribosome to insert an amino acid at the premature stop codon, rather than terminating dystrophin protein translation, may lead to *de novo* dystrophin. A small molecule drug, ataluren, has been developed as a stop codon read-through drug, and has shown variable improvement in 6-minute walk times [29]. Drug-responsive increases in dystrophin in patient muscle has not yet been demonstrated, and a recent report showed 30% of boys with DMD with a stop codon to show residual dystrophin in muscle without ataluren treatment [15]. Ataluren has been approved by the EMA since 2014.

Exon skipping, the conversion of out-of-frame to in-frame deletions, can be achieved by exon skipping (with oligonucleotides or U7 snRNPs expressing an antisense sequence) or by exon deletions (with genome editing). The majority of clinical studies of drug-induced *de novo* dystrophin in muscle have been

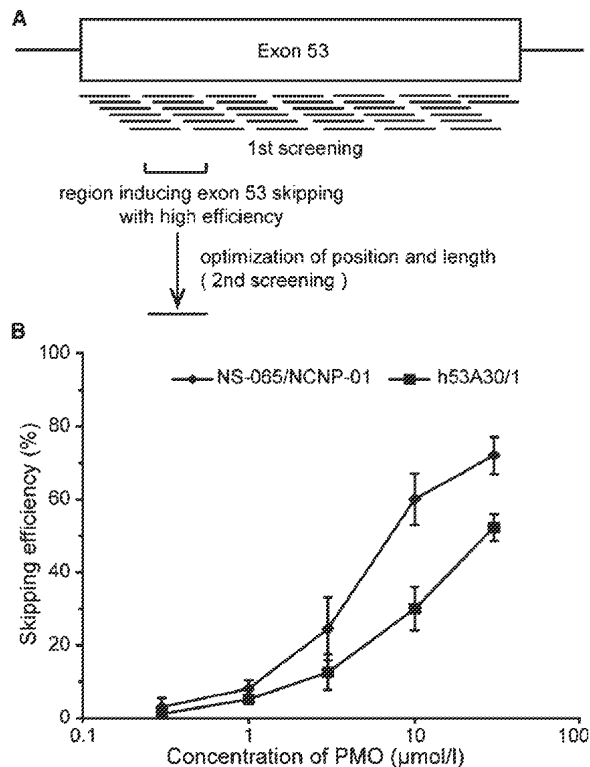


Fig. 2. Lead candidate selection for exon 53 exon skipping. Panel A: Shown is a schematic of the 38 oligonucleotides tested for strength in blocking exon 53 splicing, and the experimental approach leading to lead compound selection (NS-065/NCNP-01; viltolarsen). Panel B: Dose-response analyses shows NS-065/NCNP-01 (viltolarsen) to achieve ~70% exon skipping efficiency in cell cultures. From Watanabe et al. 2018 [62].

with exon-skipping; the removal of an additional *DMD* gene exon neighboring a patient's deletion mutation, to convert an out-of-frame *DMD* mutation to an in-frame *BMD* mutation (Fig. 1, 2). For exon-skipping, there are three different experimental approaches: oligonucleotide, DNA editing (CRISPR) and U7 snRNP-mediated splice blocking. CRISPR DNA editing approaches, while not yet in clinical trials in *DMD*, seek to modify the myofiber genomic DNA to convert a *DMD* out-of-frame to a *BMD*-like in-frame deletion. CRISPR relies on delivery of the DNA editing machinery using viral vectors. A second approach to accomplish exon-skipping is U7 snRNP-mediated blocking of splicing, similar in mechanism of action to oligonucleotide approaches. AAV vectors have been used to deliver modified U7 snRNP genes where the normal antisense part that hybridizes to histone RNA is replaced with an antisense sequence targeting (in this case) a dystrophin exon. This does not target mRNA, but pre-mRNA (like exon skipping

with oligonucleotides). A single clinical trial of AAV-mediated RNA editing (scAAV9.U7.ACCA; NCT04240314) is underway for exon 2 duplications [30]. The 3<sup>rd</sup> approach to accomplishing exon-skipping is using oligonucleotide drugs to bind to the pre-mRNA (prior to splicing) to modulate RNA splicing. These oligonucleotide approaches have used multiple chemistries for the drug, with variable success, and this is discussed further in the remainder of this text.

To explain exon skipping in more detail, oligonucleotide drugs bind to the dystrophin pre-mRNA (prior to splicing) and block the inclusion of an exon neighboring the patient's gene deletion (Figs. 1, 2). The 79 exons of the *DMD* gene often begin and end with blunt ends, where amino acids encoded by the exons are fully encoded by a triplet codon residing within the exon. For example, as shown in Fig. 1 (note gene is oriented right to left, so the bottom strand is read as encoded RNA), the end of exon 53 encodes the AAG codon for a lysine at position 2,624 (K-2624) in the dystrophin amino acid sequence and this exon 53 is spliced to the blunt end of exon 54 which itself encoded a complete codon for the following glutamic acid residue (Q-2625). However, other exons encode incomplete amino acid codons at their termini: the end of exon 52 encodes the first "C" nucleotide of isoleucine (I-2554 in red font), and requires the next 2 thymidine bases of exon 53 to complete the codon (C-TT in DNA, or CUU in RNA) (Fig. 1). Deletion mutations where remaining exons share the same reading frame are 'in-frame', and when spliced together lead to a relatively stable mRNA encoding partly functional dystrophin lacking amino acids corresponding to the deleted exons. On the other hand, remaining exons that do not share the same reading frame are 'out-of-frame', and when the remaining exons are spliced together into mRNA, a translational frame-shift is encountered by the ribosome, leading to a halt in dystrophin protein translation. Most mRNA transcripts with premature stop codons trigger a nonsense-mediated decay (NMD) mechanism that targets these out-of-frame mRNAs for degradation. With the *DMD* gene, it appears that premature stop codons may also lead to epigenomic changes in the gene, reducing mRNA expression as well [31].

An example for the viltolarsen drug targeting exon 53 of the dystrophin mRNA is diagrammed (Fig. 2). If the oligonucleotide drug hits its RNA target, the drug blocks the inclusion of the exon to which the drug is bound in the dystrophin RNA, bringing the transcript back into frame – effectively converting the *DMD*

gene mutation to a BMD-like gene mutation at the level of the mRNA.

For oligonucleotide-based exon skipping, pre-clinical studies in the *mdx* mouse model were carried out first using intramuscular injection of 2'-O-methyl phosphorothioate (2OMePS) oligonucleotides [32], and soon after using systemic delivery of both 2OMePS and phosphorodiamidate morpholino oligomer (PMO) chemistries [33, 34]. Key to success of oligonucleotide approaches is achieving adequate drug concentrations within the myofiber, so that drug can hit its pre-mRNA target in the myofiber nucleus (prior to pre-mRNA splicing) and block the splicing of the targeted exon. Measurements of myofiber delivery of oligonucleotide drugs have been done using three different methods: *in vitro* cell transfections, *in vivo* direct intramuscular injections, and *in vivo* systemic delivery (intravenous or subcutaneous). Different oligonucleotide chemistries show distinct effectiveness of myofiber delivery by these three methods, and thus show different potency in driving exon skipping depending on the assay system. Morpholino chemistry (PMO) are uncharged molecules, and are difficult to transfect into cells *in vitro*, show little or no delivery to normal myofibers by systemic delivery, but in dystrophic muscle show high level delivery and high potency in driving exon skipping [35–37]. The effective delivery of PMOs to dystrophic muscle seems to be mediated, at least in part, by myoblasts and macrophages as an intermediate to dystrophin-deficient myofibers [40]. On the other hand, 2OMePS chemistries are negatively charged, transfect well into cells *in vitro*, and can be delivered by intramuscular injection, but have not yet been shown to drive dystrophin production in patient muscle in clinical trials. Direct injection into muscle tissue destabilizes myofiber membranes near the injection site and leads to bulk delivery of any DNA or RNA payload to either normal or dystrophic muscle. Most cell types cannot recover from such overt breaches of their plasma membranes, but the enormous syncytial myofibers can recover and then retain the nucleic acids delivered by this 'brute force' approach.

To date, the highest levels of dystrophin rescue by shown by systemic delivery to skeletal muscle in mouse, dog, and human studies have been with the morpholino chemistry. A key advantage of the morpholino chemistry is that it has shown a good safety profile at very high systemic doses; up to 3.0 grams/kg in mice [38], 200 mg/kg in dogs [39], and 80 mg/kg in DMD boys [40, 41].

## CLINICAL TRIALS OF OLIGONUCLEOTIDE-INDUCED EXON SKIPPING

Exon-skipping clinical trials in DMD have been carried out with two different chemistries; 2OMePS (drisapersen), morpholino (viltolarsen, eteplirsen, golodirsen, casimersen). In addition, a locked chirality, stereopure ASO (suvodirsen) has been tested in a clinical trial, but not yet published. Here, we focus on clinical studies using 2OMePS and morpholino (PMO) chemistries for oligonucleotide drugs.

### 2OMePS (drisapersen)

The earliest exon skipping clinical trials for DMD began in 2006 and were designed to test the 2OMePS chemistry targeting exon 51 skipping (alternatively named PRO-051, GSK2402968, drisapersen) as an addition to corticosteroid standard of care treatment. In an initial open label study, 0.8 mg drisapersen was injected into the tibialis anterior muscle of 4 boys with DMD. Assessment of muscle dystrophin 28 days later showed evidence of drug-related *de novo* dystrophin expression by muscle biopsy at the injection site [42]. This promising intramuscular injection pilot study was followed by an open label, dose-finding trial of 12 DMD boys given 5 weekly subcutaneous injections, with 3 boys at each of 4 doses (0.5, 2.0, 4.0, 6.0 mg/kg), followed by a 12-week extension with all 12 boys treated with 6.0 mg/kg/wk. There was a suggestion of improvement in 6-minute walk distance, and some evidence of dystrophin mRNA splicing and dystrophin in muscle biopsies, although both appeared to be at very low levels that were difficult to distinguish from pre-treatment samples [43]. These same 12 boys were followed in a long-term extension study of weekly drisapersen for ~3.4 years (all participants also continued corticosteroid treatment). When compared to matched, steroid-treated, natural history controls, there was a suggestion of prolongation of ambulation compared to external controls [44].

These exploratory and dose-finding trials of drisapersen were then followed by two double-blind Phase II placebo-controlled studies. The first Phase II study (NCT01153932) enrolled 53 steroid-treated participants with DMD ( $7.7 \pm 1.5$  yrs) into 3 arms; placebo, intermittent drisapersen, and continuous (once weekly) drisapersen [45] (Table 1). The treatment period was 48 wks, however the primary outcome was at study midpoint (25 wks; change

in 6-minute walk test drisapersen continuous vs placebo). The continuous 6 mg/kg/wk drisapersen group showed an improvement of ~30 meters, whereas the placebo group showed a slight decline of ~5 meter ( $\Delta 35$  meters;  $p = 0.014$ ). Both groups then showed an overall decline in meters walked over the subsequent 23 wk treatment period while the 35 meter difference was maintained ( $p = 0.051$ ). Dystrophin studies of muscle biopsy were carried out, however quantitative measures of dystrophin by immunoblot were not reported.

A second placebo-controlled Phase II study (NCT01462292) of subcutaneous drisapersen administration was carried out in 51 participants with DMD randomized to 3 arms (drisapersen 3 mg/kg/wk, 6 mg/kg/wk or placebo) [46] (Table 1). For the primary outcome, 6-minute walk distance, the drisapersen 3.0 mg/kg/wk and placebo groups showed a small decline from baseline to 24 weeks, whereas the drisapersen group 6.0 mg/kg/wk showed a small improvement which did not achieve statistical significance.

The Phase 3, randomized, double-blind, placebo-controlled clinical trial, carried out from 2010–2013 at 44 sites in 19 countries, enrolled 186 boys with DMD randomized 2:1 to drisapersen vs. placebo, with a 48-wk treatment period (NCT01254019) [47]. Recruited participants were older than 5 years at entry (mean [SD] age  $8.1 \pm 2.4$  yrs), and were treated with corticosteroids for over 3 months at the time of first study drug administration. At the end of the treatment period, there was no significant clinical improvement relative to placebo. A *post-hoc* subgroup analysis of less severe participants at entry (ability to rise from floor, and 6-minute walk 300–400 meters), suggested a 35 meter reduction in decline in drisapersen group relative to placebo ( $p = 0.039$ ) [50]. Approval from the FDA in the USA was sought, but the regulatory agency noted the lack of robust evidence of efficacy, and the safety concerns of extensive injection site reactions that continued after drug cessation [48] [<https://www.fdanews.com/ext/resources/files/11-15/11-20-FDA-DMD-Briefing.pdf?1520841005>], and the program was terminated.

#### *Phosphorodiamidate morpholino (PMO)* (eteplirsen, golodirsen, viltolarsen, casimersen)

Clinical trials of the phosphorodiamidate morpholino oligonucleotide (PMO) chemistries began with a study of intramuscular injection of an exon-51

oligonucleotide (AVI-4658; eteplirsen) into a small foot muscle (extensor digitorum brevis; EDB) in 7 boys with DMD [49]. Injections were 0.09 mg ( $n = 2$ ) or 0.9 mg ( $n = 5$ ) of the morpholino oligonucleotide, with muscle biopsy 3–4 weeks after the single injection. Strong evidence of drug-related increase in both altered mRNA (skipped exon 51) and *de novo* dystrophin production were seen at the higher dose. As noted above, intramuscular injections are a robust delivery method for nucleic acids to myofibers that may be a factor unique to muscle tissue.

An open-label dose-finding study of intravenously administered eteplirsen was carried out in 19 participants with DMD (mean age 8.7 yrs) [50]. Doses tested were 0.5, 1.0, 2.0, 4.0, 10.0, and 20.0 mg/kg/wk, over a 12-week treatment period, with 2 to 4 participants per dose group (Table 1). A dose-responsive increase in dystrophin in muscle biopsies was seen with the highest dose group of 4 participants showing a mean of 4% levels by Western blot (range 0 – 7.7% increase from baseline). The authors carried out motor function assessments and did not see evidence of dose-related functional improvement, although the short treatment period and small number of subjects limited interpretation of these findings. Further analysis of the biopsies from this study showed restoration of the dystrophin-associated proteins in myofibers, consistent with the degree of dystrophin restoration [51]. An extension study was not done beyond the 12-week treatment.

A placebo-controlled dose-finding study for eteplirsen was carried out in 12 participants with DMD (mean age 8.8 years), with 4 participants randomized to placebo, 30 mg/kg/wk, or 50 mg/kg/wk for a 24-week treatment period [52] (Table 1). The primary outcome was dystrophin-positive myofibers measured by immunohistochemistry (after 12 weeks treatment for 4 patients who received 50 mg/kg and 2 patients who received placebo). A drug-related increase in dystrophin-positive myofibers was seen, but this result did not appear to be dose-responsive (30 mg/kg/wk 23.0% [range 15.9 to 29.0%]; 50 mg/kg/wk 0.8% [–9.3 to 7.4%]), although the time of treatment at the time of biopsy differed for the two groups (24 weeks for 30 mg/kg/wk; 12 weeks for 50 mg/kg/wk). There also appeared to be variability in measures of dystrophin positive myofibers, as the placebo biopsies showed appreciable variation from baseline to post-treatment (+4.5%, –5.8%, –6.5%, –8.5%). Quantitative immunoblot data were not reported. Analysis of 6-minute walk data showed an overall decline in all groups over

Table 1  
Summary of clinical trials of systemic oligonucleotide delivery for exon-skipping

Publication (clinicaltrials.gov)	Trial dates	Drug; dose groups	Delivery	# participants (Age $\pm$ SD)	Treatment period	Dystrophin (immunoblot)	6-minute walk change vs placebo
<i>2'-O-methyl oligonucleotide</i>							
Voit <i>et al.</i> 2014 (NCT01153932)	2010–2012	drisapersen; placebo, 6.0 mg/kg/wk (weekly; intermittent)	Subcutaneous	53 (7.7 $\pm$ 1.5 yrs) <sup>1</sup>	25 wks 49 wks <sup>2</sup>	Not quantitated	25 wks: +35 m ( $p=0.014$ ) <sup>49</sup> 49 wks: +35 m ( $p=0.051$ ) +27 m (ns)
McDonald <i>et al.</i> 2018 (NCT01462292)	2011–2013	drisapersen; placebo, 3.0, 6.0 mg/kg/wk	Subcutaneous	51 (7.6 $\pm$ 2.7 yrs)	24 wks	ND	
Goemens <i>et al.</i> 2017 (NCT01254019)	2010–2013	drisapersen; placebo, 6.0 mg/kg/wk	Subcutaneous	186 (8.1 $\pm$ 2.4 yrs)	48 wks	ND	+10 m (ns)
<i>phosphorodiamidate morpholino oligonucleotide</i>							
Cirak <i>et al.</i> 2011 (NCT00844597)	2008–2010	eteplirsen; 0.5, 1.0, 2.0, 4.0, 10.0, and 20.0 mg/kg/wk	Intravenous	19 (8.7 yrs)	12 wks	~4% (20.0 mg/kg; $n=4$ )	ns
Mendell <i>et al.</i> 2013 (NCT01396239)	2011–2012	eteplirsen; placebo, 30, 50 mg/kg/wk	Intravenous	12 (8.8 $\pm$ 1.2 yrs)	24 wks	24 wks: ND <sup>3</sup> 3.5 yrs: 0.9% <sup>4</sup>	–103 m (30 mg/kg) +25 m (50 mg/kg) NR
Frank <i>et al.</i> 2020 (NCT02310906)	2015–2019	golodirsen; placebo, dose escalation (4, 10, 20, 30 mg/kg/wk sequentially for 2 weeks each)	Intravenous	12 Group 1 (8.6 $\pm$ 2.1 yrs) <sup>5</sup> 13 Group 2 (8.5 $\pm$ 2.5 yrs)	24 wks	1%	
Komaki <i>et al.</i> 2018 (NCT02081625)	2013–2014	viltolarsen, 1.25, 5, or 20 mg/kg/wk	Intravenous	10 (11.0 $\pm$ 3.0 yrs)	12 wks	2% (20 mg/kg) <sup>6</sup>	NR
Clemens <i>et al.</i> 2020 (NCT02740972)	2016–2017	viltolarsen, 40, 80 mg/kg/wk	Intravenous	16 (7.4 $\pm$ 1.8 yrs)	24 wks	5.7% (40 mg/kg) 5.9% (80 mg/kg)	+28.9 m (+94.2 m vs. external control)
Komaki <i>et al.</i> 2020 (JAPIC CTI-163291)	2016–2017	viltolarsen, 40, 80 mg/kg/wk	Intravenous	16 (8.4 $\pm$ 2.0 yrs)	24 wks	1.5% (40 mg/kg) 4.8% (80 mg/kg)	–25 m (both doses)

<sup>1</sup>Placebo group was younger (6.9  $\pm$  1.2 yrs). <sup>2</sup>The treatment period was 49 weeks, but the primary outcome was change in 6-minute walk test at 24 weeks vs. placebo. <sup>3</sup>Immunoblot of a single post-treatment biopsy at 48 wks treatment was shown, but quantitations were not reported. <sup>4</sup>Participants were enrolled in an extension study, and re-biopsies after 3.5 years of treatment (Charleston *et al.* 2018). <sup>5</sup>Placebo group was mean 1.5 years younger. <sup>6</sup>Of the 4 subjects in the 20 mg/kg/wk group, one showed 8% levels of dystrophin, whereas the other 3 showed undetectable dystrophin.

the 24-week period, with the greatest decline in the 30 mg/kg/day group (−103 m vs. placebo). Subjects were enrolled in an extension study, and additional biopsies taken after 3.5 years of treatment in 11 of the participants, and this showed a mean of 0.9% post-treatment dystrophin levels [53]. This data was submitted to FDA for accelerated approval based on the surrogate biomarker of drug-related increase in dystrophin in patient muscle. The approval was granted (30 mg/kg/wk), but with considerable controversy within the FDA [54], and biomedical research community [55–57]. There are also concerns that the eteplirsen drug was not optimized for exon skipping (e.g. not potent), with alternative morpholino sequences showing over 10-fold greater potency in driving drug-induced exon skipping [58]. Eteplirsen was not approved by the European Medicines Agency (EMA) despite two attempts [59].

Golodirsen is a PMO directed against exon 53 and was first tested in a 24-week placebo-controlled, dose-escalation study of 12 participants with DMD (4 placebo; 8 golodirsen [all escalating from 4 to 30 mg/kg/wk] over the 24-week treatment period) [60] (Table 1). Participants were then enrolled into a long-term extension study (all at 30 mg/kg/wk), and an additional 13 participants with DMD who entered the 30 mg/kg/wk golodirsen treatment arm directly. The 25 golodirsen-treated participants had skeletal muscle biopsies taken at 48-weeks post-treatment. Dystrophin immunoblot analysis showed a mean of drug-related increase of 1% dystrophin. The 6-minute walk test was assessed after 2.7 years of treatment, where FDA noted “Performance on the 6-minute walk test and pulmonary function tests, with at least 144 weeks of follow-up, showed a decline from baseline; however, these results are not interpretable in the absence of a control group” [61]. Golodirsen (30 mg/kg/wk) was approved by FDA based on the surrogate biomarker of drug-related dystrophin expression.

Viltolarsen is a PMO directed against exon 53, similar to golodirsen, and directed against a target sequence on exon 53 that partially overlaps. The approach to the optimization of exon 53 skipping potency for lead compound selection for viltolarsen, as well as the pre-clinical development program, has been fully described [62]. In the initial stage of screening, 38 overlapping 25 nucleotide 2OMePS oligonucleotides were tested for potency (dose-response) in a cell culture system (Fig. 2). This defined a region between nucleotide positions 31 and 65 on the exon 53 sequence that was most effective in

blocking the inclusion of exon 53 (exon skipping). In the second stage, a series of 25 PMO oligonucleotides of varying length (15–25 nucleotides) was designed covering the position 31–65 region. This identified a 21 nucleotide PMO, named NS-065/NCNP-01 (viltolarsen), located between positions 36 and 56 that was most potent at blocking inclusion of exon 53. The effectiveness of viltolarsen was further confirmed using DMD patient-derived myogenic cells, with transfection of the cells facilitated by Endo-Porter transfection. Evidence for both efficient drug targeting of the pre-mRNA, as well as dose-responsive *de novo* dystrophin protein from the DMD patient-derived cells was seen after 3 days at a concentration of 10 mmol/L. Viltolarsen-responsive pre-mRNA exon skipping and dystrophin protein rescue was found to be dependent on drug concentration, time of drug exposure, and repeated treatment. Highest levels achieved were 80% normal levels of dystrophin rescued in patient cells.

The first human clinical study of viltolarsen was a Phase 1 dose-ranging study conducted in Japan and enrolled 10 boys with DMD (6 to 16 yrs). Study participants were treated with viltolarsen at intravenous doses of 1.25, 5, or 20 mg/kg/wk for 12 weeks [63] (Table 1). Six of the participants were treated with corticosteroids prior to and during the study, and four were not treated with corticosteroids. Seven of the 10 were non-ambulant at the initiation of treatment. Dose-responsive increases in exon skipping by RT-PCR assays of biopsy mRNA, and increased dystrophin protein production were observed. By immunoblotting, one of the four participants treated with 20 mg/kg/wk, who was also the participant with the greatest absolute dose of viltolarsen based on his body weight, showed 8% of a normal dystrophin level in the post-treatment muscle biopsy, and others showed evidence of dystrophin-positive myofibers by immunostaining.

Given the good safety profile of the PMO chemistry, the results of the Phase 1 study that had a maximum tested dose of 20 mg/kg/wk that showed a dose-dependent increase in muscle truncated dystrophin production and previous studies in mice and dogs that suggested that higher doses might be needed to drive stronger dystrophin expression, two parallel Phase II clinical trials were carried out in Japan and the US using higher doses of 40 and 80 mg/kg/wk [43, 64]. The US study was carried out by the Cooperative International Neuromuscular Research Group (CINRG), and randomized 16 steroid-treated participants with DMD to placebo [4 weeks safety

only], 40 and 80 mg/kg/wk. After 4 weeks treatment period, the participants receiving placebo were randomized to viltolarsen for the remainder of the 24-week treatment period. The primary outcome was increase in dystrophin by immunoblot from baseline to 24-weeks, with secondary outcomes of dystrophin immunostaining, RT-PCR of dystrophin mRNA, and mass spectrometry quantitation of dystrophin (orthogonal approach [65]). All biopsy analyses were done blinded. Mean drug-related increase in dystrophin for the 40 mg/kg/wk group was 5.7% [range 3.2–10.3] of normal, and for the 80 mg/kg/wk group 5.9% [range 1.1–14.4] of normal, and the dystrophin protein rescue corresponded to observed exon-skipping in dystrophin mRNA (Table 1) (Fig. 3). The immunostaining, RT-PCR and mass spectrometry methods showed significant dose-response, with approximately a 2-fold increased rescue in the 80 mg/kg/wk group compared to the 40 mg/kg/wk group (Table 2). Functional outcome measures were studied with assessments at baseline, 12-weeks and 24-weeks (tests of 6-minute walk, 10-meter run/walk, time to climb 4 stairs, time to stand from supine). All outcome measures showed mean improvement from baseline after 24-weeks treatment, and all were significant when compared to a matched, steroid-treated natural history comparator group from the CINRG DNHS study [66, 67].

A harmonized parallel study carried out in Japan recruited 16 participants with DMD randomized to 2 groups (40 mg/kg/wk; 80 mg/kg/wk) [64]. All subjects had a pre-treatment muscle biopsy, then 8 had a post-treatment biopsy after a 12-week treatment period, and the remaining 8 after a 24-week treatment period. A dose-related increase in dystrophin mRNA exon skipping was observed, as well as dose-related increase in dystrophin expression by both immunoblot and immunostaining (Table 1). The drug-related increase in dystrophin by immunoblot

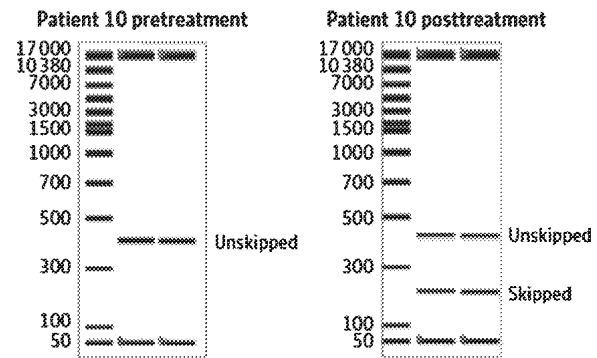


Fig. 3. Example of an RNA blot showing viltolarsen-induced exon skipping in DMD participant muscle. From Clemens et al. 2020 [40].

in the 80 mg/kg/wk group in the Japan trial (mean 4.8% normal) was similar to the findings with the same dose and treatment period in the US trial (mean 5.9% normal). Motor outcomes measures declined in both dose groups in the Japan study, in contrast to the US study, although the participants were older in the Japan study (mean 8.4 yrs) compared to US study (mean 7.4 yrs). Viltolarsen was granted accelerated approval based on dystrophin findings in both the US and Japan [64].

A PMO directed against exon 45 (casimersen) was approved by FDA in 2021. At the time of writing, there are no publications or FDA materials yet available to review the clinical trials that served as the basis for the approval.

## LESSONS LEARNED AND NEXT STEPS

From the clinical findings to date, oligonucleotide drugs based on the 2OMePS chemistry appear to lack a sufficient therapeutic index to drive adequate levels of dystrophin without dose-limiting toxicities. Interestingly, nusinersen (Spinraza) approved for

Table 2  
Orthogonal studies of dystrophin rescue by viltolarsen. From Clemens et al. 2020 [40]

Samples		Dystrophin Western blot		Mass Spectrometry	Dystrophin IF Analysis	RNA RT-PCR
Participant Cohort		Normalized myosin heavy chain	Normalized to alpha-actinin	% Dystrophin	% Dystrophin-positive fibers	% Skipped mRNA (molarity [nmol/L])
		Mean $\pm$ SD	Mean $\pm$ SD	Mean	Mean $\pm$ SD	Mean $\pm$ SD
40 mg/kg	Pre	0.3 $\pm$ 0.1	0.2 $\pm$ 0.2	0.5	1.5 $\pm$ 1.0	0.0 $\pm$ 0.0
	Post	5.7 $\pm$ 2.4	5.4 $\pm$ 2.8	2.1	14.3 $\pm$ 7.8	17.4 $\pm$ 7.2
80 mg/kg	Pre	0.6 $\pm$ 0.8	0.4 $\pm$ 0.7	0.6	1.8 $\pm$ 2.4	0.0 $\pm$ 0.0
	Post	5.9 $\pm$ 4.5	3.7 $\pm$ 2.4	4.2	34.8 $\pm$ 20.4	43.9 $\pm$ 16.7
Overall	Pre	0.4 $\pm$ 0.6	0.3 $\pm$ 0.5	0.6	1.7 $\pm$ 1.8	0.0 $\pm$ 0.0
	Post	5.8 $\pm$ 3.4	4.5 $\pm$ 2.6	3.1	24.5 $\pm$ 18.3	30.6 $\pm$ 18.5

intrathecal administration for treatment of spinal muscular atrophy is based on 2OMOE, a chemistry similar to 2OMePS, but has not run into the dose-limiting toxicities that drisapersen did. This highlights the relevance of site of administration and frequency of dosing, with apparent impacts on both efficacy and safety. The intrathecal dosing of nusinersen likely provides a more targeted delivery to motor neurons, compared to the subcutaneous delivery of drisapersen to skeletal muscle. The local delivery of nusinersen is done at a dose of 12 mg every 4 months, whereas drisapersen was dosed systemically at 6 mg/kg weekly; this likely leads to a much higher effective dose for motor neurons with a longer persistence of drug.

Oligonucleotide drugs for DMD based on the PMO chemistry (eteplirsen, golodirsen, viltolarsen, casimersen) show a broader therapeutic index, without the dose-limiting toxicities seen with other oligonucleotide chemistries. The morpholino backbone is not metabolized, and does not seem to trigger the innate immunity reactions triggered by the 2OMePS chemistries, which appears to contribute to greater safety of the PMO chemistry at higher doses (80 mg/kg/wk viltolarsen, compared to 6 mg/kg/wk drisapersen). That said, it is clear that only a very small fraction of the PMO drugs delivered by intravenous infusion reach the myofiber nucleus. Also, the mechanisms that have been defined for PMO delivery to skeletal muscle (intramuscular injection; systemic delivery via macrophages, myoblasts, and unstable myofiber membranes) are unlikely to provide dystrophin restoration for other cell types. Thus, the PMO technology may be a dystrophic muscle-specific chemistry. Multiple efforts are underway to achieve better delivery of oligonucleotides to muscle, including conjugates and muscle homing peptides.

In reviewing the experience with exon skipping to date, what are the lessons learned? First, it seems that the oligonucleotide delivery to myofibers *in vivo* remains a key variable that is relatively poorly understood. It is critical to understand this mechanism in order to optimize treatment (drug doses; frequency of dosing; mode of delivery). While studies of low levels of normal dystrophin (normal in molecular weight and biochemical composition) in some patients suggest that very low levels may have clinical benefit [15], the abnormal dystrophin (Becker-like in biochemical composition) is only partially functional. Thus, very low amounts of *de novo* dystrophin from therapeutic approaches (all biochemically abnormal) are expected to show less clinical benefit than the

same amounts of biochemically normal dystrophin. Thus, published studies of clinical correlates of low levels of normal dystrophin are likely not relevant to similarly low levels of therapeutic dystrophin [68]. Thus, it is clear that exon skipping approaches will show more compelling evidence of clinical benefit if higher levels of drug-induced dystrophin can be obtained. Much higher levels of dystrophin have been induced by oligonucleotide approaches in mice and dog models of DMD, but these have utilized much higher doses of drug than are currently utilized in human studies (up to 10-fold higher). Efforts to optimize dosing regimens may be best studied in animal models, given the many variables to be explored [69].

Most patients with DMD show little or no dystrophin, and *de novo* expression of dystrophin may invoke cell-mediated immunity, as seen in murine dystrophin exon skipping studies [70], and with AAV viral gene delivery of dystrophin in the dog model of DMD [71]. Thus, efforts to monitor anti-dystrophin antibodies, prevent the onset of cell-mediated immunity, and mitigate possible clinical relevance of anti-dystrophin antibodies will be important going forward. Studies have shown that amniotic delivery of micro-dystrophin to CXMD dog fetuses may induce immune tolerance enabling later re-delivery of micro-dystrophin by AAV vectors [72].

As noted elsewhere in this review, all therapeutic efforts to restore dystrophin in DMD patient muscle involves semi-functional, biochemically abnormal types of dystrophin, not the full-length normal protein. We cannot expect the delivery of semi-functional dystrophin to 'cure' skeletal muscle to normal muscle tissue; success is defined as a Becker-type muscle and phenotype. As such, the dystrophic milieu of skeletal muscle will continue, even as high level production of *de novo* dystrophin become increasingly successful. The dystrophic milieu has been shown to lead to short-lived AAV-derived therapeutic mRNAs [73], and the pro-inflammatory state in Becker muscle leads to induction of microRNAs that bind to the dystrophin mRNA and inhibit dystrophin protein translation [74, 75]. It is likely that polypharmacy approaches will be needed to retain and stabilize Becker-like dystrophins at multiple levels.

Finally, it is increasingly clear that the skeletal muscle phenotype of a patient with DMD changes as a function of age, with early activation of innate immunity soon after birth, but later connective tissue replacement of specific muscles at different ages that drives functional disability [76, 77]. Expressing *de novo* dystrophin in connective tissue is unlikely

to lead to any functional benefit, and some muscle groups in a boy with DMD already show connective tissue replacement even at young ages, depending on the muscle group. Thus, earlier treatment at younger ages is expected to show more clinical benefit over the disease course, assuming that the molecular pathways leading to connective tissue replacement can be slowed or stopped. Increased understanding of the transition from early successful muscle regeneration at young ages, to later unsuccessful regeneration and connective tissue replacement is critical [78].

In ending this overview, the authors wish to highlight the critical and ongoing contributions of Dr. Terence Partridge. Terry had major roles in the studies of systemic delivery to PMO drugs to the *mdx* mouse model [35, 36], showed that macrophages and myoblasts mediate delivery of PMO drugs to dystrophin-deficient myofibers [40], collaborated with the authors on the proof-of-concept systemic delivery studies of PMOs in the dog model [42], and noted that anti-dystrophin antibodies that may occur with drug-induced *de novo* dystrophin in mouse models [73]. Terry's broad contributions and fundamental insights across the development of viltolarsen and other exon skipping drugs reflect his seminal contributions to translational muscle research, as well as his endearing and highly collaborative nature.

## ACKNOWLEDGMENTS

**Disclosures:** S.T. has patents on sequences for exon skip by antisense nucleic acids as a member of NCNP together with Nippon Shinyaku. As principal inventor of these patents, S.T. is entitled to a share of royalties. S.T. discloses being an ad hoc consultant for Ono Pharmaceutical, Daiichisankyo, Asahikasei Pharma, Teijin Pharma, AGADA Biosciences, and Wave and being a member of the scientific advisory boards of Nippon Shinyaku, Taiho Pharma and Sarepta therapeutics. S.T. received speaker honoraria from Japan Health Science Foundation and Astellas Pharma and has also received research supports from Taiho Pharma, Daiichisankyo, Nippon Shinyaku, Takeda Pharmaceutical and the Noguchi Institute in the past 3 years.

Drs. Takeda, Clemens and Hoffman have been directly involved in the development of viltolarsen, and serve as consultants to the developer and marketer of viltolarsen, Nippon Shinyaku and NS Pharma. Drs. Clemens and Hoffman serve on the Board of TRiNDS LLC, a clinical contract research organization (CRO)

that aids the design, conduct and management of clinical trials in neuromuscular disease, including viltolarsen. Dr. Hoffman is co-founder of AGADA BioSciences, a CRO that facilitates drug development in neuromuscular disease, including development of viltolarsen. Dr. Hoffman is co-founder and CEO of ReveraGen BioPharma, developer of vamorolone as a potential replacement for corticosteroids in inflammatory disease. Dr. Clemens holds contracts from ReveraGen for implementation of vamorolone trials and is on the advisory board for NS Pharma, Epirium, RegenXBio and Edgewise. Dr. Clemens has grants from the National Institutes of Health, NS Pharma, Sanofi Genzyme, Amicus and Spark.

## REFERENCES

- [1] Muntoni F, Torelli S, Ferlini A. Dystrophin and mutations: One gene, several proteins, multiple phenotypes. *Lancet Neurol.* 2003;2(12):731-40.
- [2] Gao QQ, McNally EM. The Dystrophin Complex: Structure, Function, and Implications for Therapy. *Compr Physiol.* 2015;5(3):1223-39. doi: 10.1002/cphy.c140048
- [3] Pillers DA, Fitzgerald KM, Duncan NM, Rash SM, White RA, Dwinnell SJ, Powell BR, Schnur RE, Ray PN, Cibis GW, Weleber RG. Duchenne/Becker muscular dystrophy: Correlation of phenotype by electroretinography with sites of dystrophin mutations. *Hum Genet.* 1999;105(1-2):9. doi: 10.1007/s004399900111
- [4] Bucher F, Friedlander MSH, Aguilar E, Kurihara T, Krohn TU, Usui Y, Friedlander M. The long dystrophin gene product Dp427 modulates retinal function and vascular morphology in response to age and retinal ischemia. *Neurochem Int.* 2019;129:104489. doi: 10.1016/j.neuint.2019.104489
- [5] Taylor PJ, Betts GA, Maroulis S, Gilissen C, Pedersen RL, Mowat DR, Johnston HM, Buckley MF. Dystrophin gene mutation location and the risk of cognitive impairment in Duchenne muscular dystrophy. *PLoS One.* 2010;5(1):e8803. doi: 10.1371/journal.pone.0008803
- [6] Naidoo M, Anthony K. Dystrophin Dp71 and the Neuropathophysiology of Duchenne Muscular Dystrophy. *Mol Neurobiol.* 2020;57(3):1748-67. doi: 10.1007/s12035-019-01845-w
- [7] Doorenweerd N, Mahfouz A, van Putten M, Kaliyaperumal R, T' Hoen PAC, Hendriksen JGM, Aartsma-Rus AM, Verschuuren JJGM, Niks EH, Reinders MJT, Kan HE, Lelieveldt BPF. Timing and localization of human dystrophin isoform expression provide insights into the cognitive phenotype of Duchenne muscular dystrophy. *Sci Rep.* 2017;7(1):12575. doi: 10.1038/s41598-017-12981-5
- [8] Hoffman EP, Kunkel LM, Angelini C, Clarke A, Johnson M, Harris JB. Improved diagnosis of Becker muscular dystrophy by dystrophin testing. *Neurology.* 1989;39(8):1011-7. doi: 10.1212/wnl.39.8.1011
- [9] Anthony K, Cirak S, Torelli S, Tasca G, Feng L, Arechavala-Gomez V, Armaroli A, Guglieri M, Straathof CS, Verschuuren JJ, Aartsma-Rus A, Helderma-van den Enden P, Bushby K, Straub V, Sewry C, Ferlini A, Ricci E, Morgan JE, Muntoni F. Dystrophin quantification and clinical correlations in Becker muscular dystrophy:

- Implications for clinical trials. *Brain*. 2011;134(Pt 12):3547-59. doi: 10.1093/brain/awr291
- [10] Clemens PR, Niizawa G, Feng J, Florence J, D'Alessandro AS, Morgenroth LP, Gorni K, Guglieri M, Connolly A, Wicklund M, Bertorini T, Mah JK, Thangarajh M, Smith E, Kuntz N, McDonald CM, Henricson EK, Upadhyayula S, Byrne B, Manousakis G, Harper A, Bravver E, Iannaccone S, Spurney C, Cnaan A, Gordish-Dressman H; CINRG BNHS Investigators. The CINRG Becker Natural History Study: Baseline characteristics. *Muscle Nerve*. 2020;62(3):369-76. doi: 10.1002/mus.27011
  - [11] Anthony K, Arechavala-Gomez V, Ricotti V, Torelli S, Feng L, Janghra N, Tasca G, Guglieri M, Barresi R, Armadori A, Ferlini A, Bushby K, Straub U, Ricci E, Sewry C, Morgan J, Muntoni F. Biochemical characterization of patients with in-frame or out-of-frame DMD deletions pertinent to exon 44 or 45 skipping. *JAMA Neurol*. 2014;71(1):32-40. doi: 10.1001/jamaneurol.2013.4908
  - [12] de Feraudy Y, Ben Yaou R, Wahbi K, Stalens C, Stantzou A, Laugel V, Desguerre I; FILNEMUS Network, Servais L, Leturcq F, Amthor H. Very Low Residual Dystrophin Quantity Is Associated with Milder Dystrophinopathy. *Ann Neurol*. 2021;89(2):280-92. doi: 10.1002/ana.25951
  - [13] Anwar S, He M, Lim KRQ, Maruyama R, Yokota T. A Genotype-Phenotype Correlation Study of Exon Skip-Equivalent In-Frame Deletions and Exon Skip-Amenable Out-of-Frame Deletions across the *DMD* Gene to Simulate the Effects of Exon-Skipping Therapies: A Meta-Analysis. *J Pers Med*. 2021;11(1):46. doi: 10.3390/jpm11010046
  - [14] Bello L, Pegoraro E. The "Usual Suspects": Genes for Inflammation, Fibrosis, Regeneration, and Muscle Strength Modify Duchenne Muscular Dystrophy. *J Clin Med*. 2019;8(5):649. doi: 10.3390/jcm8050649
  - [15] Hufton M, Roper H. Variations in Duchenne muscular dystrophy course in a multi-ethnic UK population: Potential influence of socio-economic factors. *Dev Med Child Neurol*. 2017;59(8):837-42. doi: 10.1111/dmcn.13460
  - [16] Bushby KM, Gardner-Medwin D. The clinical, genetic and dystrophin characteristics of Becker muscular dystrophy. I. Natural history. *J Neurol*. 1993;240(2):98-104. doi: 10.1007/BF00858725. Erratum in: *J Neurol* 1993;240(7):453.
  - [17] Barp A, Bello L, Caumo L, Campadello P, Semplicini C, Lazzarotto A, Sorarù G, Calore C, Rampado A, Motta R, Stramare R, Pegoraro E. Muscle MRI and functional outcome measures in Becker muscular dystrophy. *Sci Rep*. 2017;7(1):16060. doi: 10.1038/s41598-017-16170-2
  - [18] van den Bergen JC, Wokke BH, Janson AA, van Duinen SG, Hulsker MA, Ginjaar HB, van Deutekom JC, Aartsma-Rus A, Kan HE, Verschuuren JJ. Dystrophin levels and clinical severity in Becker muscular dystrophy patients. *J Neurol Neurosurg Psychiatry*. 2014;85(7):747-53. doi: 10.1136/jnnp-2013-306350
  - [19] Wang B, Li J, Xiao X. Adeno-associated virus vector carrying human minidystrophin genes effectively ameliorates muscular dystrophy in mdx mouse model. *Proc Natl Acad Sci U S A*. 2000;97(25):13714-9. doi: 10.1073/pnas.240335297
  - [20] Sakamoto M, Yuasa K, Yoshimura M, Yokota T, Ikemoto T, Suzuki M, Dickson G, Miyagoe-Suzuki Y, Takeda S. Microdystrophin cDNA ameliorates dystrophic phenotypes when introduced into mdx mice as a transgene. *Biochem Biophys Res Commun*. 2002;293(4):1265-72. doi: 10.1016/S0006-291X(02)00362-5
  - [21] Yoshimura M, Sakamoto M, Ikemoto M, Mochizuki Y, Yuasa K, Miyagoe-Suzuki Y, Takeda S. AAV vector-mediated microdystrophin expression in a relatively small percentage of mdx myofibers improved the mdx phenotype. *Mol Ther*. 2004;10(5):821-8. doi: 10.1016/j.ymthe.2004.07.025
  - [22] Wang Z, Kuhr CS, Allen JM, Blankinship M, Gregorevic P, Chamberlain JS, Tapscott SJ, Storb R. Sustained AAV-mediated dystrophin expression in a canine model of Duchenne muscular dystrophy with a brief course of immunosuppression. *Mol Ther*. 2007;15(6):1160-6. doi: 10.1038/sj.mt.6300161
  - [23] Koo T, Okada T, Athanasopoulos T, Foster H, Takeda S, Dickson G. Long-term functional adeno-associated virus-microdystrophin expression in the dystrophic CXMDj dog. *J Gene Med*. 2011;13(9):497-506. doi: 10.1002/jgm.1602
  - [24] Yue Y, Pan X, Hakim CH, Kodippili K, Zhang K, Shin JH, Yang HT, McDonald T, Duan D. Safe and bodywide muscle transduction in young adult Duchenne muscular dystrophy dogs with adeno-associated virus. *Hum Mol Genet*. 2015;24(20):5880-90. doi: 10.1093/hmg/ddv310
  - [25] Mendell JR, Sahenk Z, Lehman K, Nease C, Lowes LP, Miller NF, Iammarino MA, Alfano LN, Nicholl A, Al-Zaidy S, Lewis S, Church K, Shell R, Cripe LH, Potter RA, Griffin DA, Pozsgai E, Dugar A, Hogan M, Rodino-Klapac LR. Assessment of Systemic Delivery of rAAVrh74.MHCK7.micro-dystrophin in Children With Duchenne Muscular Dystrophy: A Nonrandomized Controlled Trial. *JAMA Neurol*. 2020;77(9):1122-1131. doi: 10.1001/jamaneurol.2020.1484
  - [26] Willcocks RJ, Forbes SC, Walter GA, Sweeney L, Rodino-Klapac LR, Mendell JR, Vandenborne K. Assessment of rAAVrh.74.MHCK7.micro-dystrophin Gene Therapy Using Magnetic Resonance Imaging in Children With Duchenne Muscular Dystrophy. *JAMA Netw Open*. 2021;4(1):e2031851. doi: 10.1001/jamanetworkopen.2020.31851
  - [27] Weber T. Anti-AAV Antibodies in AAV Gene Therapy: Current Challenges and Possible Solutions. *Front Immunol*. 2021;12:658399. doi: 10.3389/fimmu.2021.658399
  - [28] Salabarria SM, Nair J, Clement N, Smith BK, Raben N, Fuller DD, Byrne BJ, Corti M. Advancements in AAV-mediated Gene Therapy for Pompe Disease. *J Neuromuscul Dis*. 2020;7(1):15-31. doi: 10.3233/JND-190426
  - [29] Campbell C, Barohn RJ, Bertini E, Chabrol B, Comi GP, Darras BT, Finkel RS, Flanigan KM, Goemans N, Iannaccone ST, Jones KJ, Kirschner J, Mah JK, Mathews KD, McDonald CM, Mercuri E, Nevo Y, Péréon Y, Renfroe JB, Ryan MM, Sampson JB, Schara U, Sejersen T, Selby K, Tulinius M, Vîlchez JJ, Voit T, Wei LJ, Wong BL, Elfring G, Souza M, McIntosh J, Trifillis P, Peltz SW, Muntoni F; PTC124-GD-007-DMD Study Group; ACT DMD Study Group; Clinical Evaluator Training Groups. Meta-analyses of ataluren randomized controlled trials in nonsense mutation Duchenne muscular dystrophy. *J Comp Eff Res*. 2020;9(14):973-84. doi: 10.2217/ceer-2020-0095
  - [30] Gushchina LV, Frair EC, Rohan NL, Bradley AJ, Chavan HD, Chou HJ, Eggers M, Waldrop MA, Wein N, Flanigan K. Lack of toxicity in non-human primates receiving clinically relevant doses of an AAV9.U7snRNA vector designed to induce DMD exon 2 skipping. *Hum Gene Ther*. 2021 Jan 6. doi: 10.1089/hum.2020.286
  - [31] García-Rodríguez R, Hiller M, Jiménez-Gracia L, van der Pal Z, Balog J, Adamzek K, Aartsma-Rus A, Spitali P. Premature termination codons in the *DMD* gene cause

- reduced local mRNA synthesis. *Proc Natl Acad Sci U S A*. 2020;117(28):16456-64. doi: 10.1073/pnas.1910456117
- [32] Mann CJ, Honeyman K, Cheng AJ, Ly T, Lloyd F, Fletcher S, Morgan JE, Partridge TA, Wilton SD. Antisense-induced exon skipping and synthesis of dystrophin in the mdx mouse. *Proc Natl Acad Sci U S A*. 2001;98(1):42-7. doi: 10.1073/pnas.011408598
- [33] Lu QL, Mann CJ, Lou F, Bou-Gharios G, Morris GE, Xue SA, Fletcher S, Partridge TA, Wilton SD. Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse. *Nat Med*. 2003;9(8):1009-14. doi: 10.1038/nm897
- [34] Alter J, Lou F, Rabinowitz A, Yin H, Rosenfeld J, Wilton SD, Partridge TA, Lu QL. Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. *Nat Med*. 2006;12(2):175-7. doi: 10.1038/nm1345
- [35] Hoffman EP, Bronson A, Levin AA, Takeda S, Yokota T, Baudy AR, Connor EM. Restoring dystrophin expression in duchenne muscular dystrophy muscle progress in exon skipping and stop codon read through. *Am J Pathol*. 2011;179(1):12-22. doi: 10.1016/j.ajpath.2011.03.050
- [36] Heemskerk HA, de Winter CL, de Kimpe SJ, van Kuik-Romeijn P, Heuvelmans N, Platenburg GJ, van Ommen GJ, van Deutekom JC, Aartsma-Rus A. In vivo comparison of 2'-O-methyl phosphorothioate and morpholino antisense oligonucleotides for Duchenne muscular dystrophy exon skipping. *J Gene Med*. 2009;11(3):257-66. doi: 10.1002/jgm.1288
- [37] Novak JS, Hogarth MW, Boehler JF, Nearing M, Vila MC, Heredia R, Fiorillo AA, Zhang A, Hathout Y, Hoffman EP, Jaiswal JK, Nagaraju K, Cirak S, Partridge TA. Myoblasts and macrophages are required for therapeutic morpholino antisense oligonucleotide delivery to dystrophic muscle. *Nat Commun*. 2017;8(1):941. doi: 10.1038/s41467-017-00924-7
- [38] Wu B, Lu P, Benrashid E, Malik S, Ashar J, Doran TJ, Lu QL. Dose-dependent restoration of dystrophin expression in cardiac muscle of dystrophic mice by systemically delivered morpholino. *Gene Ther*. 2010;17(1):132-40. doi: 10.1038/gt.2009.120
- [39] Yokota T, Lu QL, Partridge T, Kobayashi M, Nakamura A, Takeda S, Hoffman E. Efficacy of systemic morpholino exon-skipping in Duchenne dystrophy dogs. *Ann Neurol*. 2009;65(6):667-76. doi: 10.1002/ana.21627
- [40] Clemens PR, Rao VK, Connolly AM, Harper AD, Mah JK, Smith EC, McDonald CM, Zaidman CM, Morgenroth LP, Osaki H, Satou Y, Yamashita T, Hoffman EP; CINRG DNHS Investigators. Safety, Tolerability, and Efficacy of Viltolarsen in Boys With Duchenne Muscular Dystrophy Amenable to Exon 53 Skipping: A Phase 2 Randomized Clinical Trial. *JAMA Neurol*. 2020;77(8):982-91. doi: 10.1001/jamaneurol.2020.1264
- [41] Komaki H, Takeshima Y, Matsumura T, Ozasa S, Funato M, Takeshita E, Iwata Y, Yajima H, Egawa Y, Toramoto T, Tajima M, Takeda S. Viltolarsen in Japanese Duchenne muscular dystrophy patients: A phase 1/2 study. *Ann Clin Transl Neurol*. 2020;7(12):2393-408. doi: 10.1002/acn3.51235
- [42] van Deutekom JC, Janson AA, Ginjaar IB, Frankhuizen WS, Aartsma-Rus A, Bremmer-Bout M, den Dunnen JT, Koop K, van der Kooij AJ, Goemans NM, de Kimpe SJ, Ekhardt PF, Venneker EH, Platenburg GJ, Verschuuren JJ, van Ommen GJ. Local dystrophin restoration with antisense oligonucleotide PRO051. *N Engl J Med*. 2007;357(26):2677-86. doi: 10.1056/NEJMoa073108
- [43] Goemans NM, Tulinius M, van den Akker JT, Burm BE, Ekhardt PF, Heuvelmans N, Holling T, Janson AA, Platenburg GJ, Sipkens JA, Sitsen JM, Aartsma-Rus A, van Ommen GJ, Buyse G, Darin N, Verschuuren JJ, Campion GV, de Kimpe SJ, van Deutekom JC. Systemic administration of PRO051 in Duchenne's muscular dystrophy. *N Engl J Med*. 2011;364(16):1513-22. doi: 10.1056/NEJMoa1011367
- [44] Goemans N, Tulinius M, Kroksmark AK, Wilson R, van den Hauwe M, Campion G. Comparison of ambulatory capacity and disease progression of Duchenne muscular dystrophy subjects enrolled in the drisapersen DMD114673 study with a matched natural history cohort of subjects on daily corticosteroids. *Neuromuscul Disord*. 2017;27(3):203-213. doi: 10.1016/j.nmd.2016.11.013
- [45] Voit T, Topaloglu H, Straub V, Muntoni F, Deconinck N, Campion G, De Kimpe SJ, Eagle M, Guglieri M, Hood S, Liefwaard L, Loubakos A, Morgan A, Nakielly J, Quarcoo N, Ricotti V, Rolfe K, Servais L, Wardell C, Wilson R, Wright P, Kraus JE. Safety and efficacy of drisapersen for the treatment of Duchenne muscular dystrophy (DEMAND II): An exploratory, randomised, placebo-controlled phase 2 study. *Lancet Neurol*. 2014;13(10):987-96. doi: 10.1016/S1474-4422(14)70195-4
- [46] McDonald CM, Wong B, Flanigan KM, Wilson R, de Kimpe S, Loubakos A, Lin Z, Campion G; DEMAND V study group. Placebo-controlled Phase 2 Trial of Drisapersen for Duchenne Muscular Dystrophy. *Ann Clin Transl Neurol*. 2018;5(8):913-926. doi: 10.1002/acn3.579
- [47] Goemans N, Mercuri E, Belousova E, Komaki H, Dubrovsky A, McDonald CM, Kraus JE, Loubakos A, Lin Z, Campion G, Wang SX, Campbell C; DEMAND III study group. A randomized placebo-controlled phase 3 trial of an antisense oligonucleotide, drisapersen, in Duchenne muscular dystrophy. *Neuromuscul Disord*. 2018;28(1):4-15. doi: 10.1016/j.nmd.2017.10.004
- [48] Hilhorst N, Spanoudi-Kitrimi I, Goemans N, Morren MA. Injection site reactions after long-term subcutaneous delivery of drisapersen: A retrospective study. *Eur J Pediatr*. 2019;178(2):253-8. doi: 10.1007/s00431-018-3272-1
- [49] Kinali M, Arechavala-Gomez V, Feng L, Cirak S, Hunt D, Adkin C, Guglieri M, Ashton E, Abbs S, Nihoyannopoulos P, Garraalda ME, Rutherford M, McCulley C, Popplewell L, Graham IR, Dickson G, Wood MJ, Wells DJ, Wilton SD, Kole R, Straub V, Bushby K, Sewry C, Morgan JE, Muntoni F. Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: A single-blind, placebo-controlled, dose-escalation, proof-of-concept study. *Lancet Neurol*. 2009;8(10):918-28. doi: 10.1016/S1474-4422(09)70211-X
- [50] Cirak S, Arechavala-Gomez V, Guglieri M, Feng L, Torelli S, Anthony K, Abbs S, Garraalda ME, Bourke J, Wells DJ, Dickson G, Wood MJ, Wilton SD, Straub V, Kole R, Shrewsbury SB, Sewry C, Morgan JE, Bushby K, Muntoni F. Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: An open-label, phase 2, dose-escalation study. *Lancet*. 2011;378(9791):595-605. doi: 10.1016/S0140-6736(11)60756-3
- [51] Cirak S, Feng L, Anthony K, Arechavala-Gomez V, Torelli S, Sewry C, Morgan JE, Muntoni F. Restoration of the dystrophin-associated glycoprotein complex after exon skipping therapy in Duchenne muscular dystrophy. *Mol Ther*. 2012;20(2):462-7. doi: 10.1038/mt.2011.248

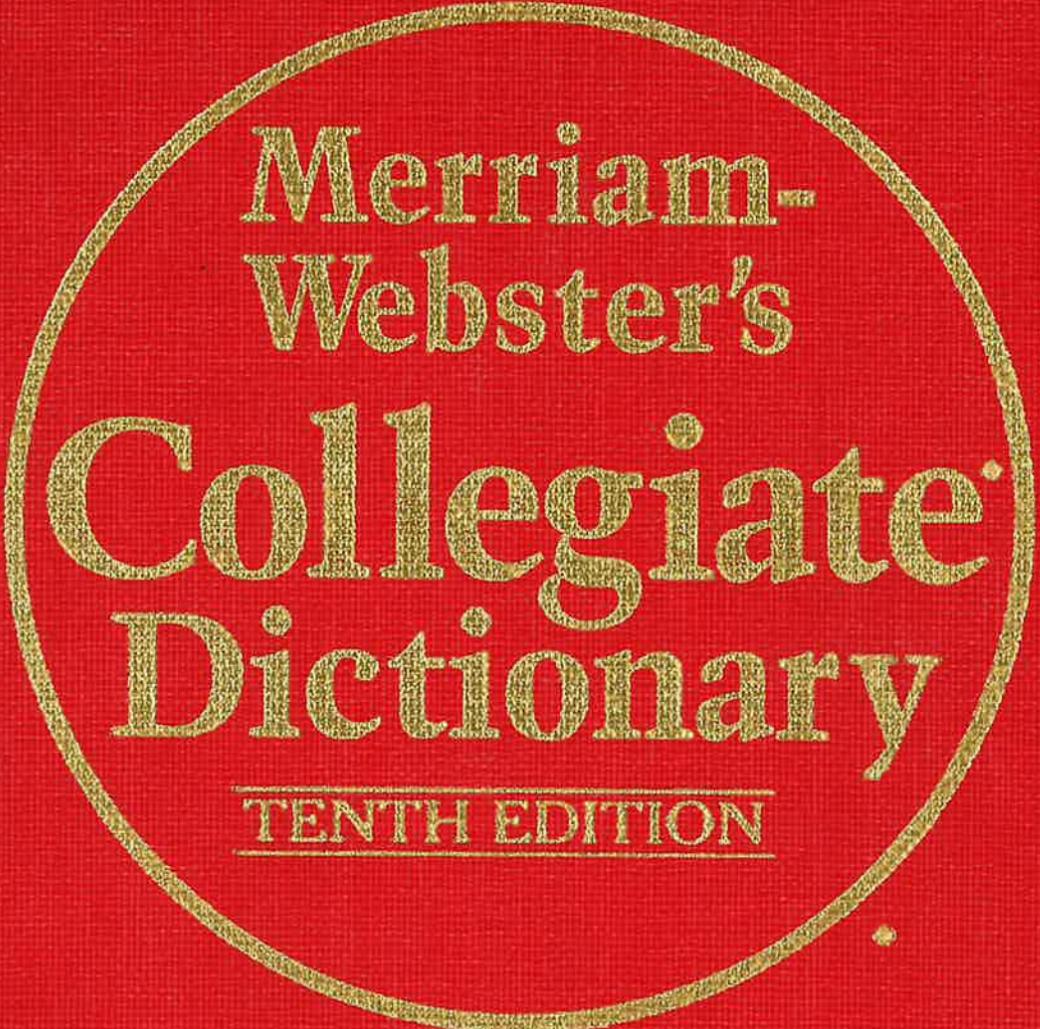
- [52] Mendell JR, Rodino-Klapac LR, Sahenk Z, Roush K, Bird L, Lowes LP, Alfano L, Gomez AM, Lewis S, Kota J, Malik V, Shontz K, Walker CM, Flanigan KM, Corridore M, Kean JR, Allen HD, Shilling C, Melia KR, Sazani P, Saoud JB, Kaye EM; Eteplirsen Study Group. Eteplirsen for the treatment of Duchenne muscular dystrophy. *Ann Neurol*. 2013;74(5):637-47. doi: 10.1002/ana.23982
- [53] Charleston JS, Schnell FJ, Dworzak J, Donoghue C, Lewis S, Chen L, Young GD, Milici AJ, Voss J, De Alwis U, Wentworth B, Rodino-Klapac LR, Sahenk Z, Frank D, Mendell JR. Eteplirsen treatment for Duchenne muscular dystrophy: Exon skipping and dystrophin production. *Neurology*. 2018;90(24):e2146-e2154. doi: 10.1212/WNL.0000000000005680
- [54] [https://www.accessdata.fda.gov/drugsatfda\\_docs/nda/2016/206488\\_summary%20review\\_redacted.pdf](https://www.accessdata.fda.gov/drugsatfda_docs/nda/2016/206488_summary%20review_redacted.pdf)
- [55] Kesselheim AS, Avorn J. Approving a Problematic Muscular Dystrophy Drug: Implications for FDA Policy. *JAMA*. 2016;316(22):2357-8. doi: 10.1001/jama.2016.16437
- [56] The Lancet. Patient need versus evidence: A balancing act. *Lancet*. 2016;388(10052):1350. doi: 10.1016/S0140-6736(16)31765-2
- [57] Unger EF, Califf RM. Regarding "Eteplirsen for the treatment of Duchenne muscular dystrophy". *Ann Neurol*. 2017;81(1):162-4. doi: 10.1002/ana.24842
- [58] Echigoya Y, Lim KRQ, Trieu N, Bao B, Miskew Nichols B, Vila MC, Novak JS, Hara Y, Lee J, Touznik A, Mamchaoui K, Aoki Y, Takeda S, Nagaraju K, Mouly V, Maruyama R, Duddy W, Yokota T. Quantitative Antisense Screening and Optimization for Exon 51 Skipping in Duchenne Muscular Dystrophy. *Mol Ther*. 2017;25(11):2561-72. doi: 10.1016/j.ymthe.2017.07.014
- [59] Aartsma-Rus A, Goemans N. A Sequel to the Eteplirsen Saga: Eteplirsen Is Approved in the United States but Was Not Approved in Europe. *Nucleic Acid Ther*. 2019;29(1):13-15. doi: 10.1089/nat.2018.0756
- [60] Frank DE, Schnell FJ, Akana C, El-Husayni SH, Desjardins CA, Morgan J, Charleston JS, Sardone V, Domingos J, Dickson G, Straub V, Guglieri M, Mercuri E, Servais L, Muntoni F; SKIP-NMD Study Group. Increased dystrophin production with golodirsen in patients with Duchenne muscular dystrophy. *Neurology*. 2020;94(21):e2270-e2282. doi: 10.1212/WNL.0000000000009233
- [61] [https://www.accessdata.fda.gov/drugsatfda\\_docs/nda/2019/211970Orig1s000SumR.pdf](https://www.accessdata.fda.gov/drugsatfda_docs/nda/2019/211970Orig1s000SumR.pdf)
- [62] Watanabe N, Nagata T, Satou Y, Masuda S, Saito T, Kitagawa H, Komaki H, Takagaki K, Takeda S. NS-065/NCNP-01: An Antisense Oligonucleotide for Potential Treatment of Exon 53 Skipping in Duchenne Muscular Dystrophy. *Mol Ther Nucleic Acids*. 2018;13:442-449. doi: 10.1016/j.omtn.2018.09.017
- [63] Komaki H, Nagata T, Saito T, Masuda S, Takeshita E, Sasaki M, Tachimori H, Nakamura H, Aoki Y, Takeda S. Systemic administration of the antisense oligonucleotide NS-065/NCNP-01 for skipping of exon 53 in patients with Duchenne muscular dystrophy. *Sci Transl Med*. 2018;10(437):eaan0713. doi: 10.1126/scitranslmed.aan0713
- [64] Komaki H, Takeshima Y, Matsumura T, Ozasa S, Funato M, Takeshita E, Iwata Y, Yajima H, Egawa Y, Toramoto T, Tajima M, Takeda S. Viltolarsen in Japanese Duchenne muscular dystrophy patients: A phase 1/2 study. *Ann Clin Transl Neurol*. 2020;7(12):2393-408. doi: 10.1002/actn.3.51235
- [65] Uaesoontrachoon K, Srinivassane S, Warford J, Mekhssian K, Montpetit H, Beauvois R, Keyhani A, Hathout Y, Yamashita T, Satou Y, Osaki H, Praest M, Moraca M, Malbasic M, Ross W, MacKinnon A, Rowsell J, Mullen A, Matyas M, Mummidivarpur S, Nagaraju K, Hoffman EP. Orthogonal analysis of dystrophin protein and mRNA as a surrogate outcome for drug development. *Biomark Med*. 2019;13(14):1209-25. doi: 10.2217/bmm-2019-0242
- [66] McDonald CM, Henricson EK, Abresch RT, Han JJ, Escolar DM, Florence JM, Duong T, Arrieta A, Clemens PR, Hoffman EP, Cnaan A; Cnrg Investigators. The cooperative international neuromuscular research group Duchenne natural history study—a longitudinal investigation in the era of glucocorticoid therapy: Design of protocol and the methods used. *Muscle Nerve*. 2013;48(1):32-54. doi: 10.1002/mus.23807
- [67] McDonald CM, Henricson EK, Abresch RT, Duong T, Joyce NC, Hu F, Clemens PR, Hoffman EP, Cnaan A, Gordish-Dressman H; CINRG Investigators. Long-term effects of glucocorticoids on function, quality of life, and survival in patients with Duchenne muscular dystrophy: A prospective cohort study. *Lancet*. 2018;391(10119):451-61. doi: 10.1016/S0140-6736(17)32160-8
- [68] Hoffman EP and Clemens PR. Letter to the Editor. *Annals Neurology in press*.
- [69] Malerba A, Thorogood FC, Dickson G, Graham IR. Dosing regimen has a significant impact on the efficiency of morpholino oligomer-induced exon skipping in mdx mice. *Hum Gene Ther*. 2009;20(9):955-65. doi: 10.1089/hum.2008.157
- [70] Vila MC, Novak JS, Benny Klimek M, Li N, Morales M, Fritz AG, Edwards K, Boehler JF, Hogarth MW, Kinder TB, Zhang A, Mazala D, Fiorillo AA, Douglas B, Chen YW, van den Anker J, Lu QL, Hathout Y, Hoffman EP, Partridge TA, Nagaraju K. Morpholino-induced exon skipping stimulates cell-mediated and humoral responses to dystrophin in mdx mice. *J Pathol*. 2019;248(3):339-51. doi: 10.1002/path.5263
- [71] Yuasa K, Yoshimura M, Urasawa N, Ohshima S, Howell JM, Nakamura A, Hijikata T, Miyagoe-Suzuki Y, Takeda S. Injection of a recombinant AAV serotype 2 into canine skeletal muscles evokes strong immune responses against transgene products. *Gene Ther*. 2007;14(17):1249-60. doi: 10.1038/sj.gt.3302984
- [72] Hayashita-Kinoh H, Yugeta N, Okada H, Nitahara-Kasahara Y, Chiyo T, Okada T, Takeda S. Intra-amniotic rAAV-mediated microdystrophin gene transfer improves canine X-linked muscular dystrophy and may induce immune tolerance. *Mol Ther*. 2015;23(4):627-37. doi: 10.1038/mt.2015.5
- [73] Dupont JB, Tournaire B, Georger C, Marolleau B, Jeanson-Leh L, Ledevin M, Lindenbaum P, Lecomte E, Cogné B, Dubreil L, Larcher T, Gjata B, Van Wittenberghe L, Le Guiner C, Penaud-Budloo M, Snyder RO, Moullier P, Léger A. Short-lived recombinant adeno-associated virus transgene expression in dystrophic muscle is associated with oxidative damage to transgene mRNA. *Mol Ther Methods Clin Dev*. 2015;2:15010. doi: 10.1038/mtm.2015.10
- [74] Fiorillo AA, Heier CR, Novak JS, Tully CB, Brown KJ, Uaesoontrachoon K, Vila MC, Ngheim PP, Bello L, Kornegay JN, Angelini C, Partridge TA, Nagaraju K, Hoffman EP. TNF- $\alpha$ -Induced microRNAs Control Dystrophin Expression in Becker Muscular Dystrophy. *Cell Rep*. 2015;12(10):1678-90. doi: 10.1016/j.celrep.2015.07.066
- [75] Kinder TB, Heier CR, Tully CB, Van der Muelen JH, Hoffman EP, Nagaraju K, Fiorillo AA. Muscle Weakness in Myositis: MicroRNA-Mediated Dystrophin Reduction in a Myositis Mouse Model and Human Muscle Biopsies. *Arthritis Rheumatol*. 2020;72(7):1170-1183. doi: 10.1002/art.41215

S358

*S. Takeda et al. / Exon-Skipping in Duchenne Muscular Dystrophy*

- [76] Chen YW, Nagaraju K, Bakay M, McIntyre O, Rawat R, Shi R, Hoffman EP. Early onset of inflammation and later involvement of TGFbeta in Duchenne muscular dystrophy. *Neurology*. 2005;65(6):826-34. doi: 10.1212/01.wnl.0000173836.09176.c4
- [77] Rooney WD, Berlow YA, Triplett WT, Forbes SC, Willcocks RJ, Wang DJ, Arpan I, Arora H, Senesac C, Lott DJ, Tennekoon G, Finkel R, Russman BS, Finanger EL, Chakraborty S, O'Brien E, Moloney B, Barnard A, Sweeney HL, Daniels MJ, Walter GA, Vandenborne K. Modeling disease trajectory in Duchenne muscular dystrophy. *Neurology*. 2020;94(15):e1622-e1633. doi: 10.1212/WNL.00000000000009244
- [78] Uezumi A, Ito T, Morikawa D, Shimizu N, Yoneda T, Segawa M, Yamaguchi M, Ogawa R, Matev MM, Miyagoe-Suzuki Y, Takeda S, Tsujikawa K, Tsuchida K, Yamamoto H, Fukada S. Fibrosis and adipogenesis originate from a common mesenchymal progenitor in skeletal muscle. *J Cell Sci*. 2011;124(Pt 21):3654-64. doi: 10.1242/jcs.086629

# EXHIBIT 51



Merriam-  
Webster's  
Collegiate  
Dictionary  
TENTH EDITION



**A GENUINE MERRIAM-WEBSTER**

The name *Webster* alone is no guarantee of excellence. It is used by a number of publishers and may serve mainly to mislead an unwary buyer.

*Merriam-Webster*<sup>TM</sup> is the name you should look for when you consider the purchase of dictionaries or other fine reference books. It carries the reputation of a company that has been publishing since 1831 and is your assurance of quality and authority.

Copyright © 2000 by Merriam-Webster, Incorporated

Philippines Copyright 2000 by Merriam-Webster, Incorporated

Library of Congress Cataloging in Publication Data  
Main entry under title:

Merriam-Webster's collegiate dictionary. — 10th ed.  
p. cm.  
Includes index.  
ISBN 0-87779-708-0 (unindexed : alk. paper). — ISBN 0-87779-709-9  
(indexed : alk. paper). — ISBN 0-87779-710-2 (deluxe indexed : alk. paper).  
— ISBN 0-87779-707-2 (laminated cover, unindexed).  
I. English language—Dictionaries. I. Merriam-Webster, Inc.  
PE1628.M36 1998 97-41846  
423—dc21 CIP

Merriam-Webster's Collegiate<sup>®</sup> Dictionary, Tenth Edition principal copyright 1993

COLLEGIATE is a registered trademark of Merriam-Webster, Incorporated

All rights reserved. No part of this book covered by the copyrights hereon may be reproduced or copied in any form or by any means—graphic, electronic, or mechanical, including photocopying, taping, or information storage and retrieval systems—without written permission of the publisher.

Made in the United States of America

2728293031RT:WC00

# Contents

Preface	6a
Explanatory Chart	8a
Explanatory Notes	10a
The English Language in the Dictionary	23a
Guide to Pronunciation	31a
Abbreviations in This Work	36a
Pronunciation Symbols	38a
<b>A Dictionary of the English Language</b>	<b>1</b>
Abbreviations and Symbols for Chemical Elements	1375
Foreign Words and Phrases	1387
Biographical Names	1393
Geographical Names	1439
Signs and Symbols	1529
A Handbook of Style	1533
Index	1557

**2whack**  
**whack**  
**whack**  
**whack**

with the  
shrunk  
what a  
about t

other  
curve

<sup>4</sup>when  
 <trou  
 —Lau  
 when-

1341

# EXHIBIT 52

## The Chicago Manual of Style *Online*

### 6: Punctuation

#### 6.19: Serial commas

Items in a series are normally separated by commas (but see 6.60). When a conjunction joins the last two elements in a series of three or more, a comma—known as the serial or series comma or the Oxford comma—should appear before the conjunction. Chicago strongly recommends this widely practiced usage, blessed by Fowler and other authorities (see [bibliog. 1.2](#)), since it prevents ambiguity. If the last element consists of a pair joined by *and*, the pair should still be preceded by a serial comma and the first *and* (as in the last two examples below).

She posted pictures of her parents, the president, and the vice president.

Before heading out the door, he took note of the typical outlines of sweet gum, ginkgo, and elm leaves.

I want no ifs, ands, or buts.

Paul put the kettle on, Don fetched the teapot, and I made tea.

Their wartime rations included cabbage, turnips, and bread and butter.

Ahmed was configuring updates, Jean was installing new hardware, and Alan was running errands and furnishing food.

If the sentence continues beyond the series, add a comma only if one is required by the syntax of the surrounding sentence.

Apples, plums, and grapes can all be used to make wine.

*but*

Apples, plums, and grapes, available at most large grocery stores, can all be used to make wine.

In the rare case where the serial comma does not prevent ambiguity, it may be necessary to reword. In the following example, the repetition of *and* makes it clear that Lady Gaga is not the writer's mother (and see the examples at the end of this paragraph). In the second example, "Lady Gaga" might be read as an appositive (see 6.28).

I thanked my mother and Lady Gaga and Madonna.

*not*

I thanked my mother, Lady Gaga, and Madonna.

Note that the phrase *as well as* cannot substitute for *and* in a series of items.

The team fielded one Mazda, two Corvettes, and three Bugattis, as well as a battered Plymouth Belvedere.

*not*

The team fielded one Mazda, two Corvettes, three Bugattis, as well as a battered Plymouth Belvedere.

In a series whose elements are all joined by conjunctions, no commas are needed unless the elements are long and delimiters would be helpful.

Would you prefer Mendelssohn or Schumann or Liszt?

You can turn left at the second fountain and right when you reach the temple, or left at the third fountain and left again at the statue of Venus, or in whatever direction Google sends you.

# EXHIBIT 53

**IN THE UNITED STATES DISTRICT COURT  
FOR THE DISTRICT OF DELAWARE**

NIPPON SHINYAKU CO., LTD.,

Plaintiff,

v.

SAREPTA THERAPEUTICS, INC.,

Defendant.

C.A. No. 21-1015 (GBW)

SAREPTA THERAPEUTICS, INC.,

Defendant/Counter-Plaintiff,

v.

NIPPON SHINYAKU CO., LTD.  
and NS PHARMA, INC.

Plaintiff/Counter-Defendants.

**REPLY DECLARATION OF CY A. STEIN, M.D., Ph.D.**

## Table of Contents

	<u>Page</u>
I. INTRODUCTION .....	4
II. TASK SUMMARY AND MATERIALS CONSIDERED.....	4
III. SKILLED ARTISAN.....	5
IV. DISPUTED CLAIM TERMS OF THE WILTON PATENTS.....	6
A. Term 1a: “a base sequence”; Term 1b: “a target region”; and Term 1c: “exon 53 of the human dystrophin pre-mRNA” .....	6
1. Term 1a: “a base sequence” .....	6
a. The Claim Language Supports Sarepta’s Construction .....	6
b. The Claim Language Does Not Support NS’s Construction .....	9
c. Other Intrinsic Evidence Supports Sarepta’s Construction, Not NS’s Construction.....	11
2. Term 1b: “a target region” .....	13
a. Sarepta’s Construction Conforms to How a Skilled Artisan Would Have Understood This Term.....	13
b. Dr. Hastings’ Indefiniteness Arguments Ignore the Context of How the Term Is Used in the Claims .....	14
c. Dr. Hastings’ Indefiniteness Positions Are Not Relevant.....	16
3. Term 1c: “exon 53 of the human dystrophin pre-mRNA” .....	18
a. Sarepta’s Construction Conforms to the Intrinsic Evidence and the General Knowledge in the Art .....	18
b. Dr. Hastings’ Indefiniteness Positions Are Contrary to the Intrinsic and Extrinsic Evidence .....	18
B. Term 1: “antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA” .....	21
C. Term 3: “in which uracil bases are thymine bases” .....	22
1. Dr. Hastings Arbitrarily Re-Writes the Claims.....	23

2.	Dr. Hastings’ Discussion of the Specification and Prosecution History Do Not Support Her Construction .....	26
a.	The Specification .....	26
b.	The Prosecution History of the ’722 Application .....	27
3.	Dr. Hastings’ Extrinsic Evidence Does Not Support Her Construction.....	28
4.	Dr. Hastings’ “Theoretical” Examples Ignore NS’s Product.....	31
D.	Term 2: “the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)” .....	32
1.	The Annealing Site Phrase Identifies Nucleotides +23 to +69 .....	32
2.	Dr. Hastings’ Indefiniteness Positions Are Unreasonable.....	35

I, Cy A. Stein, M.D., Ph.D., declare as follows:

## **I. INTRODUCTION**

1. I have been retained by Sarepta Therapeutics, Inc. (“Sarepta”) as an expert in the above-captioned matter. I understand that Sarepta has asserted claims 1-2 of U.S. Patent No. 9,994,851 (“the ’851 patent”; Ex. 1); claims 1-2 of U.S. Patent No. 10,227,590 (“the ’590 patent”; Ex. 2); and claims 1-2 of U.S. Patent No. 10,266,827 (“the ’827 patent”; Ex. 3) (collectively, the “Wilton patents”) against Nippon Shinyaku Co., Ltd. and NS Pharma, Inc. (collectively, “NS”). I have provided an Opening Declaration, which is incorporated by reference in its entirety (“Op. Decl.”). My qualifications, compensation and prior testimony, and task summary are described in my Opening Declaration, Sections II-IV. My curriculum vitae is attached as **Appendix A** to my Opening Declaration. I submit this declaration on behalf of Sarepta in response to the Declaration of Michelle L. Hastings, Ph.D., dated February 6, 2023 (“Hastings Decl.”).

## **II. TASK SUMMARY AND MATERIALS CONSIDERED**

2. I have considered the Hastings Declaration and cited exhibits. I have also considered the materials cited in this declaration as well as those listed in **Appendix C**.<sup>1</sup> To the extent I am provided with additional documents or information, including any subsequent expert declarations in this case, I reserve the right to modify, supplement, and/or expand my opinions based on the new information. In addition to these materials, I may consider additional documents and information in forming any supplemental opinions.

---

<sup>1</sup> The materials considered in my Opening Declaration are summarized in **Appendix B** attached to my Opening Declaration. To avoid any confusion, I have listed the materials additionally considered in this declaration in **Appendix C**.

### III. SKILLED ARTISAN<sup>2</sup>

3. Dr. Hastings agrees that individuals holding a Ph.D. in chemistry, biochemistry, cell biology, genetics, molecular biology, or an equivalent, and having several years of experience with antisense oligonucleotides for inducing exon skipping, would qualify as a skilled artisan with respect to the Wilton patents. Hastings Decl. ¶20. However, she maintains that the definition of a skilled artisan should also include individuals with an M.D., M.S., B.S., and/or B.A. in molecular biology, biochemistry, or a related area, and relevant experience. *Id.* ¶20–21.

4. I agree that a person with an M.D. degree could qualify as a skilled artisan if that the person also possesses several years of experience *designing* and using exon skipping antisense oligonucleotides and related therapies. In my opinion, a medical doctor who merely treats patients with related therapies would not qualify as a skilled artisan because she would lack the necessary educational background and experience.

5. Similarly, I disagree that a person with an M.S., B.S., and/or B.A. would qualify as a skilled artisan. As I summarized in my Opening Declaration, the claimed invention of the Wilton patents concerns exon skipping antisense oligonucleotides and their use for treating Duchenne muscular dystrophy. Op. Decl. ¶¶18–24; *see id.* ¶¶27–41 (state of the art). The level of education and training afforded by an M.S., B.S., and/or B.A. would likely be insufficient for the subject matter of the Wilton patents, as exon-skipping is highly technical and requires additional education and experience.

---

<sup>2</sup> Dr. Hastings uses the term “person of ordinary skill in the art” or “POSA” as a synonym of the term “skilled artisan” used in my Opening Declaration and in this declaration. *See* Hastings Decl. ¶¶19–21.

6. Regardless of which level of skill is applied, it is my opinion that Sarepta’s proposed constructions for the disputed terms are correct and should be adopted. As explained below, Dr. Hastings’ contrary constructions and indefiniteness arguments suffer from numerous flaws.

#### IV. DISPUTED CLAIM TERMS OF THE WILTON PATENTS

##### A. Term 1a: “a base sequence”; Term 1b: “a target region”; and Term 1c: “exon 53 of the human dystrophin pre-mRNA”

Terms	Sarepta’s Proposed Construction	NS’s Proposed Construction
“a base sequence” (Term 1a)	<i>Not indefinite with respect to Terms 1b and 1c</i>	any sequence of bases that is part of the antisense oligonucleotide
“exon 53 of the human dystrophin pre-mRNA” (Term 1c)	<i>No construction needed in light of inter alia, the intrinsic evidence and the knowledge of a person of ordinary skill in the art</i>	<i>Indefinite</i>
“a target region” (Term 1b)	To the extent construction is needed, Sarepta proposes that each phrase should be given its plain and ordinary meaning, i.e., “a linear sequence of bases” (Term 1a), “the pre-mRNA transcribed from exon 53 of the human dystrophin gene” (Term 1c), and “a segment of the pre-mRNA” (Term 1b)	<i>Indefinite</i>

##### 1. Term 1a: “a base sequence”

###### a. The Claim Language Supports Sarepta’s Construction

7. Read in context, the term “a base sequence” refers to “a linear sequence of bases” of the claimed antisense oligonucleotide as a whole, which is consistent with Sarepta’s construction. *See* Op. Decl. ¶¶50–51, 67–69. The claims refer to an “antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA.” Ex. 1 (the ’851 patent) at claim 1. Reading the term “base sequence” in this context, a skilled artisan would understand that the base

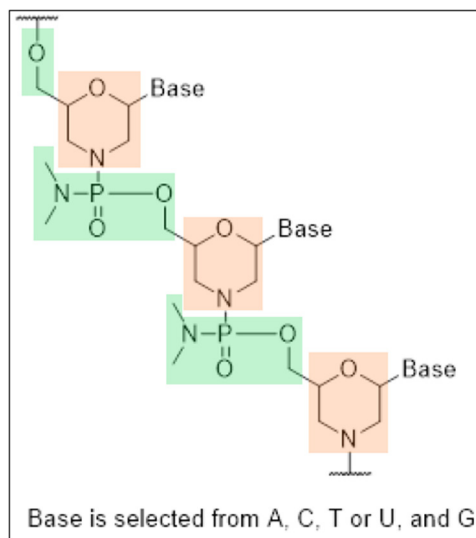
sequence of the claimed antisense oligonucleotide is a group of 20 to 31 bases, arranged in a particular order that achieves 100% complementarity to the target region. This comports with common usage in the art: while antisense oligonucleotides may have a varying number of bases, each antisense oligonucleotide is described as having *one* base sequence regardless of the number of bases. *See* Op. Decl. ¶¶53–54.

8. Dr. Hastings states that Sarepta’s construction is inconsistent with the term “comprising” in the claims. Hastings Decl. ¶¶33, 44. While I am not a lawyer, I understand that “comprising” in patent law means that the listed elements are essential but that other elements may be added. *See id.* ¶33 (“including but not limited to”). Sarepta’s construction is consistent with the use of “comprising” because it requires “a base sequence” that is 100% complementary, but also allows other elements to be present in the claims.<sup>3</sup>

9. As depicted in Figure 3 of my Opening Declaration (and reproduced as **Figure 1** below), the chemical structure of a morpholino oligomer includes bases and other components such as inter-nucleotide phosphate linkages (highlighted in green) and morpholine rings (highlighted in orange). Op. Decl. ¶40, Figure 3. Sarepta’s construction requires that all of the bases in the antisense oligonucleotide are 100% complementary to the target region, but also allows for inclusion of these additional components, consistent with the term “comprising.”

---

<sup>3</sup> Based on the same rationale, Dr. Hastings contends that the Patent Office’s statement describing an antisense oligonucleotide as *including* a nucleobase sequence supports NS’s construction. *See* Hastings Decl. ¶¶48–49. I disagree. The statement reflects the common usage of the term “base sequence” in the art: (1) referring to all bases present in an antisense oligonucleotide that are complementary to its target region and (2) allowing for other elements to be present. Op. Decl. ¶57; Ex. 17 at 3 (the Patent Office: “an *oligonucleotide* that includes a *nucleobase sequence* that is complementary to a *portion of a particular pre-mRNA exon*”) (emphasis added).



**Figure 1.** Annotated Structure of Morpholino Oligomer  
(Adapted from Figure 3 of Op. Decl.)

10. Dr. Hastings states that Sarepta's construction renders the claim language "comprising a base sequence" superfluous. Hastings Decl. ¶35. I disagree. Sarepta's construction clarifies that the bases in the antisense oligonucleotide have a specific order. For example, the claims require "20 to 31 bases," but that limitation alone does not convey that those bases must be arranged in an order that achieves 100% complementarity to the target region. The following two base sequences are illustrative:

**Base Sequence #1:** CTG AAG GTG TTC TTG TAC TTC ATC C

(H53A(+23+47) with uracil bases substituted with thymine bases)

**Base Sequence #2:** CTG AAG GTG TTC TTT TTT AAG CCC C

(contains the same bases as Base Sequence #1 but in a different order)

11. Both sequences contain 25 bases, meeting the limitation of "20 to 31 bases." But only Base Sequence #1 falls within the scope of the claims because the linear order of bases is 100% complementary to the target region. Sarepta's construction requires that the linear order of

bases in the claimed antisense oligonucleotide—not just any order—must achieve 100% complementarity.

12. Dr. Hastings raises other points, none of which account for the context of the claims. For example, she maintains that Sarepta’s construction is inconsistent with the definition of “comprising” in the specification: “the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.” *Id.* ¶¶33 n.1, 41. But as discussed above, because Sarepta’s construction allows for other components to be present in the claimed antisense oligonucleotide, it is consistent with the definition of “comprising” in the specification. Moreover, if Dr. Hastings is reading the definition to mean that the claims encompass additional bases (e.g., “integers”), then she is incorrect because the claims already fix the number of bases included in the antisense oligonucleotide as “20 to 31 bases.” Ex. 1 (the ’851 patent) at claim 1. Dr. Hastings also states that because the claims treat “antisense oligonucleotide” and “base sequence” as separate terms, a skilled artisan would understand that “base sequence” is a subsidiary portion of the “antisense oligonucleotide.” Hastings Decl. ¶36. But given that an antisense oligonucleotide includes several, distinct components (such as morpholino rings and inter-nucleotide phosphate linkages), the claims necessarily treat the “base sequence” as a subsidiary term defining part of the antisense oligonucleotide. Listing them as two separate terms does *not* suggest that the antisense oligonucleotide has multiple “base sequences,” potentially including a base sequence that is not 100% complementary.

**b. The Claim Language Does Not Support NS’s Construction**

13. Dr. Hastings contends that NS’s construction—“any sequence of bases that is part of the antisense oligonucleotide”—should be adopted instead. *Id.* ¶32. Using the term “part” in an ambiguous manner, NS’s construction allows for the claimed base sequence to cover “part” of the claimed antisense oligonucleotide in terms of bases, i.e., the claimed antisense oligonucleotide

can contain multiple, smaller base sequences, some of which may be 100% complementary to the target region and the others that may not be. As explained, I disagree because NS's construction conflicts with the plain language of the claims and other limitations in the claims. Op. Decl. ¶¶68. The claims expressly require that the base sequence "is 100% complementary to consecutive bases of [the] target region." Ex. 1 (the '851 patent) at claim 1. Nothing in the claims subdivides the base sequence of the antisense oligonucleotide into multiple distinct base sequences.

14. Dr. Hastings appear to arrive at NS's construction by reading other claim terms in an illogical way. In particular, she reads "consecutive bases" of the "target region" to refer to a subset of bases present in the target region rather than all bases present in the target region. *See* Hastings Decl. ¶34 ("[T]he 'base sequence' must be '100% complementary' to *some* portion of the 'target region' that is consecutive.") (emphasis added). With that interpretation, Dr. Hastings then reasons that consequently, the claimed base sequence can also be 100% complementary to that subset of bases, not all the bases, in the target region. *Id.* That is incorrect. The term "consecutive bases" clarifies that the target region is a contiguous portion (i.e., segment) of exon 53 of the human dystrophin pre-mRNA. Nothing in the claims supports Dr. Hastings' reading of this "consecutive bases" term to refer to only a subset of bases in the target region or the "base sequence" to be complementary to only a "portion" of the target region.

15. Had the inventors intended for each claimed antisense oligonucleotide to include multiple base sequences, they would have written the claims as such. For example, the inventors could have stated that the claimed antisense oligonucleotides comprised a portion having a base sequence that was 100% complementary and a portion having a base sequence that was not. Instead, they required the claimed antisense oligonucleotide to comprise "a base sequence that is 100% complementary to consecutive bases of [the] target region." Ex. 1 at claim 1. Consistent

with the common use of the term “base sequence,” the plain reading of the claims supports that the claimed antisense oligonucleotide has one base sequence, not plural base sequences as Dr. Hastings suggests.

16. The consequences of adopting NS’s proposed construction highlight its flaws. As Dr. Hastings admits, under NS’s construction, the claimed base sequence can include some bases that are not complementary to the target region. *See* Hastings Decl. ¶34 (“[T]he ‘base sequence’ must be ‘100% complementary’ to *some* portion of the ‘target region’ that is consecutive.”) (emphasis added). If adopted, NS’s construction would make the 100% complementarity limitation meaningless, as the claims would cover antisense oligonucleotides that contain both complementary and non-complementary bases.

**c. Other Intrinsic Evidence Supports Sarepta’s Construction, Not NS’s Construction**

17. Dr. Hastings relies on two portions of the specification as evidence that the term “base sequence” does not apply to the entire antisense oligonucleotide. *Id.* ¶¶37–40. In my opinion, neither supports NS’s proposed construction.

18. Dr. Hastings first relies on a portion of the specification stating that “complementarity” requires only a sufficient degree of complementarity to the target region, not 100% complementarity. *Id.* ¶¶38–39 (citing Ex. 1 (the ’851 patent) at 25:26–28). The claims, however, require that the base sequence of the antisense oligonucleotide is “100% complementary” to its corresponding target region. Ex. 1 (the ’851 patent) at claim 1. Because the claims require “a base sequence that is 100% complementary,” it is irrelevant that the specification also discloses the possibility of using antisense oligonucleotides with less than 100% complementarity.

19. The related discussion in the specification further highlights the flaws in Dr. Hasting’s reasoning. Under NS’s construction, the amount of non-complementary bases that a

claimed antisense oligonucleotide could have is substantial. For example, an antisense oligonucleotide of 31 bases could have 12 bases derived from SEQ ID NO: 195 (which would be 100% complementary) and 19 bases that are completely unrelated to the dystrophin gene (which would have 0% complementary). In other words, the antisense oligonucleotide as a whole would have 12 complementary bases of 31 total, or less than 40%. But the specification expressly cautions against using antisense oligonucleotides with insufficient complementarity “to avoid non-specific binding of the antisense compound to non-target sequences.” *Id.* at 25:28–38.

20. Dr. Hastings also relies on the specification’s disclosure of “weasel” compounds, including those listed in Table 1C. Hastings Decl. ¶¶40, 43. These “weasel” compounds are not relevant to the claims. The claims are limited to antisense oligonucleotides of “20 to 31 bases,” whereas each of the exemplified weasel compounds in Table 1C contains from 47 bases to 85 bases. Ex. 1 (the ’851 patent) at Table 1C. Indeed, the only weasel compound related to exon 53 is over 75 nucleotides in length, includes non-complementary linker nucleotides, and targets both exons and introns.<sup>4</sup> *Id.* (“H53A(+23+47)-AA-H53A(+150+175)-AA-H53D(+14-07)”). Further, the specification describes these weasel compounds as “two or more antisense oligonucleotide molecules” joined together. *Id.* at 4:56–62. In contrast, the claims are directed to *an* antisense oligonucleotide. *Id.* at claim 1.

21. Dr. Hastings also relies on two statements of the patent applicant from the prosecution history of the ’851 patent. Hastings Decl. ¶¶45–47. These statements do not use the term “a base sequence.” *See* Ex. 22 (Prosecution History Excerpt of the ’851 patent) at SRPT-VYDS-0004785, SRPT-VYDS-0004789. Read in context, they do not support NS’s proposed

---

<sup>4</sup> As I explained in my Opening Declaration, the nomenclature known in the art assigns nucleotide coordinates on an intron (i.e., outside of exon 53) with a negative number. Op. Decl. ¶41.

construction. In the first statement, the applicant discussed a prior art antisense oligonucleotide called h53AON1, which is an “18-mer oligonucleotide having a sequence identical to three consecutive bases of SEQ ID NO: 195.” *Id.* at SRPT-VYDS-0004785. The applicant simply explained that the prior art antisense oligonucleotide had a base sequence of 18 bases, three of which overlapped with bases found in SEQ ID NO: 195. *Id.* at SRPT-VYDS-0004786 (the applicant explaining that the prior art antisense oligonucleotide had only three consecutive bases of SEQ ID NO: 195). The applicant did not state that h53AON1 had multiple base sequences.

22. In the second statement, the applicant summarized the Examiner’s position that a skilled artisan would have synthesized and tested “longer oligonucleotides containing within them the sequence known to have the desired activity.” *Id.* at SRPT-VYDS-0004789. In context, it is clear the applicant was referring to adding bases to an existing base sequence to create a new base sequence. *Id.* (the applicant explaining that “the Office failed to provide a reason why the skilled artisan would lengthen” the prior art antisense oligonucleotide). These prosecution statements do not support NS’s construction that would allow a single antisense oligonucleotide to have multiple base sequences, only one of which is complementary.

**2. Term 1b: “a target region”**

**a. Sarepta’s Construction Conforms to How a Skilled Artisan Would Have Understood This Term**

23. As discussed in my Opening Declaration, I agree with Sarepta’s construction of the term “target region” as a “segment of the pre-mRNA.” *See* Op. Decl. ¶¶48, 67. The claims require that the base sequence of the claimed antisense oligonucleotide is “100% complementary to consecutive bases of a *target region* of exon 53 of the human dystrophin pre-mRNA.” Ex. 1 (the ’851 patent) at claim 1 (emphasis added). Read in context, a skilled artisan would understand the term “target region” to mean a segment in exon 53 of the human dystrophin pre-mRNA to which

the base sequence of the claimed antisense oligonucleotide is intended to bind. Indeed, the claims define the target region positionally as a segment of exon 53, for example by requiring at least 12 consecutive bases of SEQ ID NO: 195 (complementary to nucleotides +23 to +47 of exon 53; Ex. 1 at Table 1A) and 100% complementarity. As Dr. Hastings noted, the specification “repeatedly” uses this term to refer to “the particular annealing site” of the antisense oligonucleotide (i.e., the specific region to which the antisense oligonucleotide is intended to bind). Hastings Decl. ¶¶58 (citing Ex. 1 (the ’851 patent) at 4:44–46, 23:38–45, 32:31–36); *see* Op. Decl. ¶48 (also citing, e.g., Ex. 1 (the ’851 patent) at 24:61–25:11).

24. This is also consistent with how the term is used in the art. Op. Decl. ¶¶53–56. Dr. Hastings, for example, has used the term in the same way in several of her publications. Ex. 48 (Havens 2013) at 250 (Dr. Hastings in 2013: “[antisense oligonucleotides] are short oligonucleotides, typically 15–25 bases in length, which are the reverse complement sequence of a specific RNA transcript *target region*”) (emphasis added); Ex. 49 (Havens 2016) at 6550 (Dr. Hastings in 2016: “all [antisense oligonucleotides] make use of short nucleic acids that specifically base-pair to a *targeted sequence*”) (emphasis added).

**b. Dr. Hastings’ Indefiniteness Arguments Ignore the Context of How the Term Is Used in the Claims**

25. Dr. Hastings states that the term “target region” is indefinite because the term could be understood to mean: (1) “identifying the exon generally being targeted” or (2) “refer[ring] to the particular motifs or regulatory regions on a pre-mRNA transcript being targeted.” Hastings Decl. ¶54. I disagree that a skilled artisan would have considered these meanings as reasonable.

26. (1) the exon being targeted: A skilled artisan would not have read the term “target region” in this manner because the claims of the Wilton patents already identify the exon generally being targeted, i.e., exon 53. *See* Ex. 1 (the ’851 patent) at claim 1 (“a target region of exon 53”).

27. The '851 patent further highlights the unreasonableness of this interpretation. The claims of the '851 patent state “wherein the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69).” As discussed in my Opening Declaration, this annealing site phrase identifies nucleotides +23 to +69 of exon 53 of the human dystrophin pre-mRNA.<sup>5</sup> Op. Decl. ¶¶85. It would be nonsensical to refer to exon 53 generally as the target region, given that the annealing site further identifies a region within exon 53.

28. (2) the motifs or regulatory regions on a pre-mRNA transcript being targeted: A skilled artisan would not have equated the term “target region” with any particular motif or regulatory region present in exon 53 of the human dystrophin pre-mRNA for several reasons. First, the claims do not reference any particular motif or regulatory region, such as a splice donor site, splice acceptor site, or exonic splicing enhancer element. Ex. 1 (the '851 patent) at claim 1. Second, a skilled artisan familiar with exon skipping would have known that the “target region” of an antisense oligonucleotide does not necessarily coincide with a particular motif or regulatory region. Third, the specification explains that targeting these motifs may or may not induce exon skipping. *See, e.g., id.* at 24:12–20 (“[T]argeting the acceptor site of exon 23 or several internal domains was not found to induce any consistent exon 23 skipping.”), 24:21–22 (“In other exons . . . , masking the donor splice site did not induce any exon skipping.”); *see also id.* at 4:17–22 (“Simply directing the antisense oligonucleotides to motifs presumed to be crucial for splicing is no guarantee of the efficacy of that compound in a therapeutic setting.”).

---

<sup>5</sup> Dr. Hastings disagrees with my interpretation of the annealing site phrase. Hastings Decl. ¶¶92. But even under her alternative interpretation, the annealing site phrase still identifies a portion of exon 53 of the human dystrophin pre-mRNA. It is therefore inconsistent with her interpretation that the “target region” would be understood as the entire exon.

29. Dr. Hastings points to select statements using the word “target” in the specification to support these constructions. Hastings Decl. ¶¶55–60, 63. But those statements merely identify certain exons or motifs that a skilled artisan could explore using antisense oligonucleotides. *See, e.g.*, Ex. 1 (the ’851 patent) at 24:21–25 (“exons targeted for removal”), 3:67–4:3 (antisense oligonucleotides were designed to “target[] the acceptor region of the mouse dystrophin pre-mRNA”), 25:12–17 (sites “involved in mRNA splicing” as “preferred target site(s)” for exploration). The same applies to the select statements from the prosecution history that Dr. Hastings identifies. Hastings Decl. ¶¶65–66; Ex. 22 (Prosecution History Excerpt of the ’851 patent) at SRPT-VYDS-0004793 (“[T]here is great variability for different targets and exons.”), SRPT-VYDS-0004794 (discussing antisense oligonucleotides that “target exon 50”). None of these statements uses the term “target region” in the same context as it’s used in the claims. As Dr. Hastings acknowledges, these “*different* contexts in which the shared specification describes ‘targeting’ pre-mRNA” (*see* Hastings Decl. ¶62) do not inform how a skilled artisan would have understood the term “target region” in the claims.

**c. Dr. Hastings’ Indefiniteness Positions Are Not Relevant**

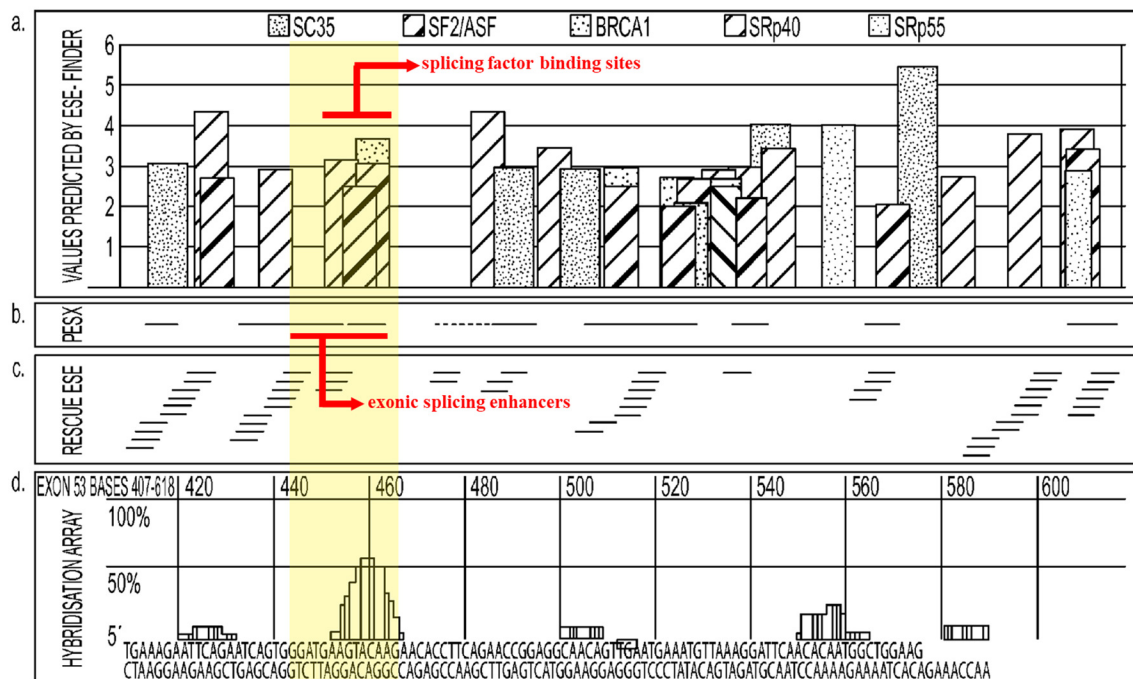
30. I understand that Sarepta has asserted that NS’s Viltepso<sup>®</sup> (viltolarsen) product infringes the Wilton patents. *See generally* Ex. 31 (Viltolarsen Label). For the purpose of this declaration, I have been instructed to consider whether NS’s Viltepso<sup>®</sup> (viltolarsen) product meets the “target region” limitation of the Wilton patents under Sarepta’s construction and Dr. Hastings’ two other constructions.

31. In my opinion, NS’s Viltepso<sup>®</sup> (viltolarsen) product meets the “target region” limitation under each construction.

32. Under Sarepta’s construction, NS’s Viltepso<sup>®</sup> (viltolarsen) product meets the “target region” limitation at least because it is 100% complementary to a segment of the pre-

mRNA transcribed from exon 53 of the human dystrophin gene. Ex. 50 (Takeda 2021) at S351 (“positions 36 and 56” in exon 53 of the human dystrophin pre-mRNA are the “target” region of viltolarsen).

33. Under Dr. Hastings’ other constructions, NS’s Viltepso® (viltolarsen) product also meets the “target region” limitation because: (1) it is directed to an exon of interest, exon 53, and (2) it is directed to motifs and regulatory regions in exon 53, as illustrated in **Figure 2** below. *Id.* (“Viltolarsen is a PMO directed against exon 53.”); *see also* Ex. 38 (the ’601 patent) at Fig. 1 (reproduced below with positions 36 to 56 identified in yellow), 4:3–24 (explaining that Fig. 1(a) and (b) show the predicted locations bound by various splicing factors, and predicted locations for exonic splicing enhancers and exonic splicing silencers, respectively).



**Figure 2.** Motifs and Regulatory Regions in Exon 53  
(adapted from Fig. 1 of Ex. 38 (the ’601 patent))

34. While Dr. Hastings discusses hypothetical differences in claim scope under her constructions, she offers no opinion as to whether those constructions affect whether NS’s

Viltepso<sup>®</sup> (viltolarsen) product meets the “target region” limitation. *See* Hastings Decl. ¶¶60–61.

In my opinion, they do not.

**3. Term 1c: “exon 53 of the human dystrophin pre-mRNA”**

**a. Sarepta’s Construction Conforms to the Intrinsic Evidence and the General Knowledge in the Art**

35. As discussed in my Opening Declaration, I agree with Sarepta’s construction that the term “exon 53 of the human dystrophin pre-mRNA” means “the pre-mRNA transcribed from exon 53 of the human dystrophin gene.” *See* Op. Decl. ¶¶47, 49, 67. This conforms to the general knowledge in the relevant art. A protein is made from a gene through three general biological processes: transcription (from the gene to a pre-mRNA), splicing (from the pre-mRNA to an mRNA), and translation (from the mRNA to the protein). *Id.* ¶¶27–32. The exon skipping strategy uses an antisense oligonucleotide to alter the splicing process. *Id.* The antisense oligonucleotide is designed to bind to a segment of the pre-mRNA transcribed from a gene of interest and alter splicing events such that an exon of interest is “skipped.” *Id.* ¶¶36–38.

**b. Dr. Hastings’ Indefiniteness Positions Are Contrary to the Intrinsic and Extrinsic Evidence**

36. Dr. Hastings does not appear to disagree with my analysis or Sarepta’s construction. *See* Hastings Decl. ¶¶69–80. But she states that a skilled artisan “would immediately find it unclear whether the claim term ‘exon 53 of the human dystrophin pre-mRNA’ refers to exon 53 from wildtype pre-mRNA or patient’s mutated pre-mRNA.” *Id.* I disagree for the reasons discussed in my Opening Declaration. Op. Decl. ¶65. The sequence of each exon of the human dystrophin gene, including exon 53, was well known in the art. *Id.* ¶34; Hastings Decl. ¶70 (Dr. Hastings acknowledging the same). Consistent with this knowledge in the art, the claims refer to “exon 53 of *the* human dystrophin pre-mRNA,” not exon 53 of *a* human dystrophin pre-mRNA.

Op. Decl. ¶¶65. A skilled artisan reading the claims would understand that the term refers to *the* sequence known in the art, i.e., the wild-type sequence of exon 53. *Id.*

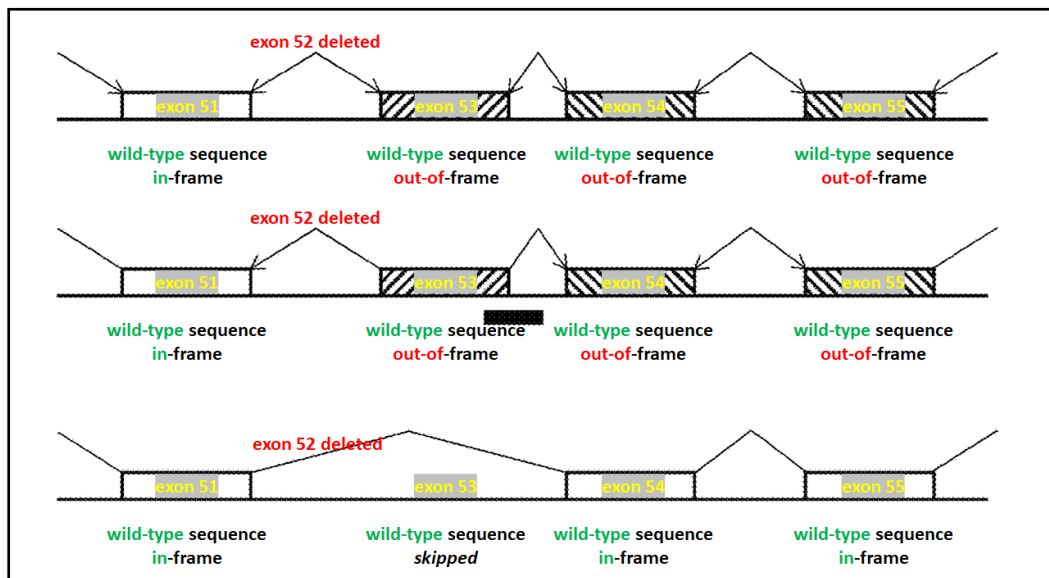
37. Dr. Hastings nevertheless states that the term could be referring to a mutated sequence because: (1) the specification and the prosecution history of the Wilton patents refer to experiments conducted with “mutated cell lines” (Hastings Decl. ¶¶76–78); and (2) the term is used in the claims of the ’827 patent, which require treating a patient with Duchene muscular dystrophy who “has a mutation of the DMD gene that is amenable to exon 53 skipping” (*id.* ¶¶71–75). In my opinion, neither reason supports Dr. Hastings’ construction.

38. First, the “mutated cell lines” referenced in the specification and the prosecution history carry mutations in exons *other than* exon 53 in cells derived from species *other than* humans. *See* Ex. 1 (the ’851 patent) at 3:22–40 (“*exon 23* of mutated dystrophin in the mdx *mouse* mutant”); *id.* (“myoblasts from the mdx *mouse*” used to test an antisense oligonucleotide to “dystrophin *intron 22* . . . cause[d] skipping of *the mutated exon* [i.e., *exon 23*]”); Ex. 22 (Prosecution History Excerpt of the ’851 patent) at SRPT-VYDA-0004791–92 (discussing Ex. 19); Ex. 19 (Mann 2002) at Fig. 1 (summarizing tested antisense oligonucleotides directed to *exon 23 and intron 23* of “*mouse* dystrophin”). These mutated cell lines from other species would not inform what the term “*exon 53 of the human* dystrophin pre-mRNA” means to a skilled artisan.

39. In contrast, as Dr. Hastings acknowledges, experiments discussed in the specification conducted with the *human* dystrophin gene always involve targeting the wild-type sequence of an exon of interest. Hastings Decl. ¶¶76–77 (citing Ex. 1. at 3:8–21, 32:48–55); *see* Ex. 1 (the ’851 patent) at 3:8–21 (“wild-type pre-mRNA”), 32:48–55 (“normal primary myoblast cultures”). From my review, I did not identify any experiment disclosed in the specification where an antisense oligonucleotide was designed to bind to a mutated sequence of an exon of interest of

the human dystrophin pre-mRNA. Nor does Dr. Hastings identify any such experiment in her declaration.

40. While Dr. Hastings also relies on Figure 2 in the specification to support the concept of targeting a “mutated” exon, her interpretation of Figure 2 is incorrect. Hastings Decl. ¶72. As discussed in my Opening Declaration, Duchenne muscular dystrophy results from mutations in the dystrophin gene that disrupt the reading frame of the dystrophin mRNA. Op. Decl. ¶35. Figure 2 of the Wilton patents illustrates that concept. As shown below in **Figure 3**, I used the deletion of exon 52 as an example. The hatched boxes represent exons that follow the deletion of exon 52 and are out-of-frame. Being out-of-frame does not mean that those exons carry mutated sequences—they still have the wild-type sequences but cannot be read in-frame to produce a functional dystrophin protein. Using an antisense oligonucleotide, an exon (in this case, exon 53) is skipped such that the frame of the remaining exons is restored. Figure 2 does not represent targeting a “mutated exon” as Dr. Hastings describes. It represents targeting the wild-type sequence of an exon of interest, like exon 53, that can be skipped to remedy the mutation.



**Figure 3.** Annotated Figure 2 of the Wilton Patents

41. Second, the '827 method of treatment patent's use of this term would not change its plain meaning to a skilled artisan. Dr. Hastings ignores that the same claim term is used in the claims of two Wilton patents that do *not* reference patients with DMD.

42. Moreover, the claims of the Wilton patents do not reference any particular exon 53 mutation(s), and Dr. Hastings identifies no prior art reference reporting a mutated pre-mRNA sequence of exon 53 that is amenable by exon 53 skipping. I am not aware of any such reference. *See, e.g.*, Ex. 4 (Aartsma-Rus 2002) at Table 3 (listing “the DMD-causing mutations” potentially correctable by exon 53 skipping antisense oligonucleotides, none of which involves a mutation in exon 53). Dr. Hastings' indefiniteness opinions therefore appear to be based on a hypothesis that a mutated pre-mRNA sequence of exon 53 *may* exist in a patient, which *may* be known to a skilled artisan. *See* Hastings Decl. ¶79 (a patient's mutated pre-mRNA for exon 53 “may or may not differ from the wildtype sequence”). Even if such patients exist, a skilled artisan would not understand the term “exon 53 of the human dystrophin pre-mRNA” to be referring to a mutated form of the gene.

**B. Term 1: “antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA”**

Sarepta's Proposed Construction	NS's Proposed Construction
<p><i>Not indefinite</i></p> <p><i>No construction needed in light of inter alia, the intrinsic evidence and the knowledge of a person of ordinary skill in the art</i></p> <p>To the extent construction is needed, Sarepta proposes that the phrase should be given its plain and ordinary meaning, i.e.,</p> <p>“antisense oligonucleotide that has 20 to 31 bases, which collectively form a linear sequence that is 100% complementary to a</p>	<p><i>Indefinite</i></p> <p>Or, in the alternative:</p> <p>“antisense oligonucleotide with 20 to 31 bases that includes any sequence of bases that is part of the antisense oligonucleotide that are 100%</p>

Sarepta’s Proposed Construction	NS’s Proposed Construction
segment of the pre-mRNA transcribed from exon 53 of the human dystrophin gene”	complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA”

43. In my Opening Declaration, I explained why this antisense oligonucleotide phrase should be interpreted in its entirety. Op. Decl. ¶¶44–62. In response, Dr. Hastings asserts that, even if the entire phrase is construed, Sarepta’s construction should be rejected because it omits “comprising” and “consecutive bases of.” Hastings Decl. ¶¶82–89.

44. I disagree. Sarepta’s construction does not omit “comprising” because it allows for other components of the claimed antisense oligonucleotide to be included in the claims, including morpholino rings, inter-nucleotide phosphate linkages, and a 5’ cap. *See supra* ¶¶8–12. In other words, Dr. Hastings ignores that antisense oligonucleotides contain more than just a linear sequence of bases (a base sequence). Sarepta’s construction is not “converting” the claims to require 100% complementarity—that is a core feature of the claims.

45. Sarepta’s construction also does not omit “consecutive bases of” because the construction requires “100% complementarity to a segment of the pre-mRNA,” conveying the consecutive nature of the bases arranged in the target region. *See* Op. Decl. ¶¶45, 48, 52, 59. In other words, under Sarepta’s individual constructions or its collective construction, the base sequence in the antisense oligonucleotide must be 100% complementary to consecutive bases in a segment of the pre-mRNA.

**C. Term 3: “in which uracil bases are thymine bases”**

Sarepta’s Proposed Construction	NS’s Proposed Construction
<i>Not indefinite</i>  <i>No construction needed in light of inter alia, the intrinsic evidence and the knowledge of a person of ordinary skill in the art</i>	<i>Indefinite</i>

Sarepta’s Proposed Construction	NS’s Proposed Construction
To the extent construction is needed, Sarepta proposes that the phrase should be given its plain and ordinary meaning, i.e., “the antisense oligonucleotide has thymine bases instead of uracil bases”	

46. In my Opening Declaration, I explained that a skilled artisan would understand this thymine bases phrase to modify the claimed antisense oligonucleotide in its entirety based on the claim language, the specification, the prosecution history of the related ’722 application, and general knowledge in the art. Op. Decl. ¶¶71–83. In response, Dr. Hastings proposes her own construction—“namely that the uracil bases of the specifically recited SEQ ID NO: 195 are replaced with thymine bases in the claims.” Hastings Decl. ¶101. Dr. Hastings states that because a skilled artisan would not have known which construction to adopt with reasonable certainty, this phrase is indefinite. *Id.* ¶102.

47. As discussed below, I disagree. A skilled artisan would have known with reasonable certainty how to interpret the claim language.

#### 1. Dr. Hastings Arbitrarily Re-Writes the Claims

48. In my Opening Declaration, I provided the following analysis of the claims (reproduced as **Figure 4** as below), illustrating that each “wherein” and “in which” clause independently and equally applies to the claimed antisense oligonucleotide as a whole. Dr. Hastings agrees that “this is one reasonable way [a skilled artisan] could understand and break down the claim language itself.” Hastings Decl. ¶121.

1. An antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA,

wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195),

in which uracil bases are thymine bases,

wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, and

wherein the antisense oligonucleotide induces exon 53 skipping;

or a pharmaceutically acceptable salt thereof.

**Figure 4.** Structure of the Claims of the Wilton Patents (adapted from Paragraph 72 of Op. Decl.)

49. But Dr. Hastings states that there is another way to format the claims, as reproduced below in **Figure 5**, and that this reformatting “demonstrate[s] how a [skilled artisan] would reasonably understand the phrase . . . to modify the listing of SEQ ID NO: 195 that immediately precedes the phrase.” Hastings Decl. ¶¶123–124.

1. An antisense oligonucleotide of 20 to 31 bases comprising a base sequence that is 100% complementary to consecutive bases of a target region of exon 53 of the human dystrophin pre-mRNA,

wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195), in which uracil bases are thymine bases,

wherein the antisense oligonucleotide is a morpholino antisense oligonucleotide, and

wherein the antisense oligonucleotide induces exon 53 skipping;

or a pharmaceutically acceptable salt thereof.

**Figure 5.** Reformatting of the Claims of the Wilton Patents by Dr. Hastings (adapted from Paragraph 123 of Hastings Decl.)

50. Dr. Hastings identifies three purported reasons for interpreting the claims in this manner: (1) the thymine bases phrase uses “in which” unlike the “wherein” clauses; (2) the

thymine bases phrase does not specify what is being modified; and (3) the language of the thymine bases phrase suggests a pre-existing series of bases that includes uracil bases. *Id.* ¶¶125–128. None of these reasons support Dr. Hastings’ reformatting of the claims.

51. First, there is no meaningful distinction between the word “wherein” and the phrase “in which” because they are known synonyms. Ex. 51 (Merriam-Webster Dictionary) at 1341 (“wherein” defined as “in which”). This understanding is reflected in the prosecution history of the ’722 application, where both the Examiner and the applicant referred to the thymine bases phrase as “wherein uracil bases are thymine bases.” Ex. 20 (Prosecution History Excerpt of U.S. Application No. 15/273,772) at SRPT-VYDS-0094154–55, SRPT-VYDS-0094181. A skilled artisan would not have treated the “in which” clause and the “wherein” clauses differently because they are synonyms and were used interchangeably during prosecution before the Patent Office.

52. Second, Dr. Hastings ignores the larger context of the claims. As explained, the claims of the Wilton patents set forth four separate characteristics of the claimed antisense oligonucleotide using the “wherein” and “in which” clauses. Op. Decl. ¶73; *see supra* **Figure 4** . A skilled artisan would have recognized that each clause is directed to and characterizes the claimed antisense oligonucleotide.

53. Third, the thymine bases phrase does not assume any pre-existing series of bases. Rather, it simply instructs a skilled artisan to use thymine bases instead of uracil bases when constructing the claimed antisense oligonucleotides. In my opinion, if the phrase were intended to refer only to the bases in SEQ ID NO: 195, there would have been no reason to separate SEQ ID NO: 195 and the disputed phrase with a comma. Dr. Hastings offers no explanation for ignoring this comma.

54. None of the statements made by Dr. Hastings justify deviating from the structure and plain meaning of the claims.

**2. Dr. Hastings' Discussion of the Specification and Prosecution History Do Not Support Her Construction**

**a. The Specification**

55. Dr. Hastings states that the specification does not “clarify the ambiguity in claim interpretation.” Hastings Decl. ¶129. She states that the specification contemplates both types of antisense oligonucleotides, i.e., those with uracil or thymine bases only and those potentially containing both. *Id.* ¶¶130–137. As an initial matter, I disagree that there is any “ambiguity” in claim interpretation for the reasons discussed above. Further, in my opinion, the specification supports Sarepta’s construction, not NS’s construction.

56. As Dr. Hastings appears to acknowledge, *none* of the exemplified antisense oligonucleotides in the specification contain a mixture of uracil bases and thymine bases. *See id.* ¶¶130–132. But she dismisses these antisense oligonucleotides because they are “exemplary.” *Id.* I disagree. As explained in my Opening Declaration, Table 1A and its legend (for “morpholinos, these U bases may be shown as ‘T’”) of the Wilton patents are instructive because they are consistent with the general practice in the art of making antisense oligonucleotides with uracil or thymine bases—but not both. Op. Decl. ¶¶80–83. They guide a skilled artisan to follow the same general practice. *Id.* ¶¶74–75.

57. That the specification elsewhere contemplates antisense oligonucleotides with a mixture of different modifications does not change my opinion. *See* Hastings Decl. ¶¶133–136. Indeed, nothing in the specification teaches using mixtures of uracil and thymine bases, as the passage Dr. Hastings focuses on simply discusses the use of chemically modified bases without mentioning whether a single oligonucleotide could contain a mixture of those chemically modified

bases and unmodified bases. *See id.* ¶134 (citing Ex. 1 (the '851 Patent) at 27:35–39). The claims, as structured, instruct a skilled artisan to use thymine bases instead of uracil bases in making the claimed antisense oligonucleotides. *See supra* ¶¶48–54. A skilled artisan would not have interpreted the claims to allow mixtures of uracil and thymine bases.

**b. The Prosecution History of the '722 Application**

58. The prosecution history of the '722 application reinforces that the thymine bases phrase refers to the claimed antisense oligonucleotide as a whole. Dr. Hastings attempts to dismiss these statements, asserting that (1) they refer to whether the prior art disclosed substituting uracil bases with thymine bases at all, not the location of the substitution; and (2) the statements are grammatically incorrect because they do not follow a “parallel structure.” Hastings Decl. ¶¶138–144. I respectfully disagree.

59. The statements are quite clear. In each instance, as highlighted in blue below, the applicant expressly referenced the “pending *claims*” or the “*claimed* antisense oligonucleotide,” and explained that the antisense oligonucleotide contained uracil bases rather than thymine bases.

Specifically, the pending claims are drawn to an antisense oligonucleotide having the following elements: (i) 25 bases comprising a base sequence that is 100% complementary to 25 consecutive bases of exon 53 of the human dystrophin pre-mRNA; (ii) 20 consecutive bases of SEQ ID NO: 193; (iii) uracil bases are thymine bases; (iv) the antisense oligonucleotide is a morpholino; (v) the antisense oligonucleotide induces exon 53 skipping; and (vi) the antisense oligonucleotide is chemically linked to a polyethylene glycol chain.

**Figure 6.** Annotated Excerpt #1 from the '722 Application Prosecution History  
(Ex. 20 (Prosecution History Excerpt of U.S. Application No. 15/273,772)  
at SRPT-VYDS-0094179)

Further, none of the cited references teach or suggest combining the elements to result in the claimed antisense oligonucleotide. Specifically, there is no teaching or suggestion to generate an antisense oligonucleotide of 25 bases, wherein the antisense oligonucleotide comprises 20 consecutive bases of SEQ ID NO: 193, and wherein uracil bases are thymine bases, and wherein the antisense oligonucleotide is a morpholino, and wherein the resulting antisense oligonucleotide induces exon 53 skipping of the human dystrophin pre-mRNA.

**Figure 7.** Annotated Excerpt #2 from the '722 Application Prosecution History (Ex. 20 (Prosecution History Excerpt of U.S. Application No. 15/273,772) at SRPT-VYDS-0094181)

60. Further, the applicant listed each and every clause in parallel, reinforcing that the thymine bases clause is an independent clause modifying the claimed antisense oligonucleotide. In **Figure 6**, each of the clauses is sequentially numbered (highlighted in green), indicating a parallel structure. And in **Figure 7**, each clause was distinguished using the phrase “and wherein” (highlighted in pink), again indicating a parallel structure. These excerpts demonstrate that the applicant considered the thymine bases phrase and the other listed phrases as separate clauses, each modifying the claimed antisense oligonucleotide as a whole.

### **3. Dr. Hastings’ Extrinsic Evidence Does Not Support Her Construction**

61. Dr. Hastings cites select publications as evidence that “oligonucleotides using both thymine and uracil bases exist and have been studied.” Hastings Decl. ¶106; *see id.* ¶¶107–116 (citing Exs. 24, 38–41). Those publications do not support her contention.

62. Exhibit 24: This is a review article published by Drs. Summerton and Weller in 1997. *See id.* ¶¶111–112. As Dr. Hastings noted, the article discusses assembling a morpholino oligomer using “genetic bases (adenine, cytosine, guanine, and thymine or uracil).” Ex. 24 (Summerton 1997(a)) at 188 (emphasis added). The statement refers to thymine and uracil bases as alternatives—it does not suggest assembling morpholino oligomers with mixtures of both. Notably, the antisense oligonucleotide exemplified in this article only used uracil bases, not a

mixture of uracil and thymine bases. *Id.* at 190 (“22-mer of the sequence 5’-GCUCGCAGACUUGUCCAUCAU”).

63. Exhibit 39: Exhibit 39 appears to be a printout from the Gene Tools, LLC company website. *See* Hastings Decl. ¶113. Based on the website, Dr. Hastings contends that “before 1998, Gene Tools sold morpholino oligonucleotides having uracil bases, but after 1998 Gene Tools sold morpholino oligonucleotides having thymine bases.” *Id.* The website says nothing about whether a mixture of uracil and thymine bases was ever used by Gene Tools, which is consistent with my opinion that a skilled artisan would have made antisense oligonucleotides with one or the other, but not both.

64. Exhibit 38: This is a U.S. patent issued December 27, 2011, claiming priority to two provisional applications filed in 2008 and 2009, respectively. Ex. 38 (the ’601 patent) at items (45) & (60); *see* Hastings Decl. ¶¶107–109, 114. I understand that a claim term of a patent must be interpreted from the perspective of a skilled artisan in the pertinent art *at the time of the invention*. Op. Decl. ¶15. The same applies to the definiteness inquiry. *Id.* ¶17. As such, it is unclear how this exhibit relates to what a skilled artisan would have known *at the time of the invention*, i.e., June 28, 2005. *Id.* ¶25.

65. Nevertheless, Dr. Hastings reproduces the following three sequences from Exhibit 38 and notes that the patent mentions using “all thymines, all uracils or a combination of the two” in place of X in each sequence. Hastings Decl. ¶¶107–108 (citing Ex. 38 (the ’601 patent) at 3:55–63, 6:12–19).

```

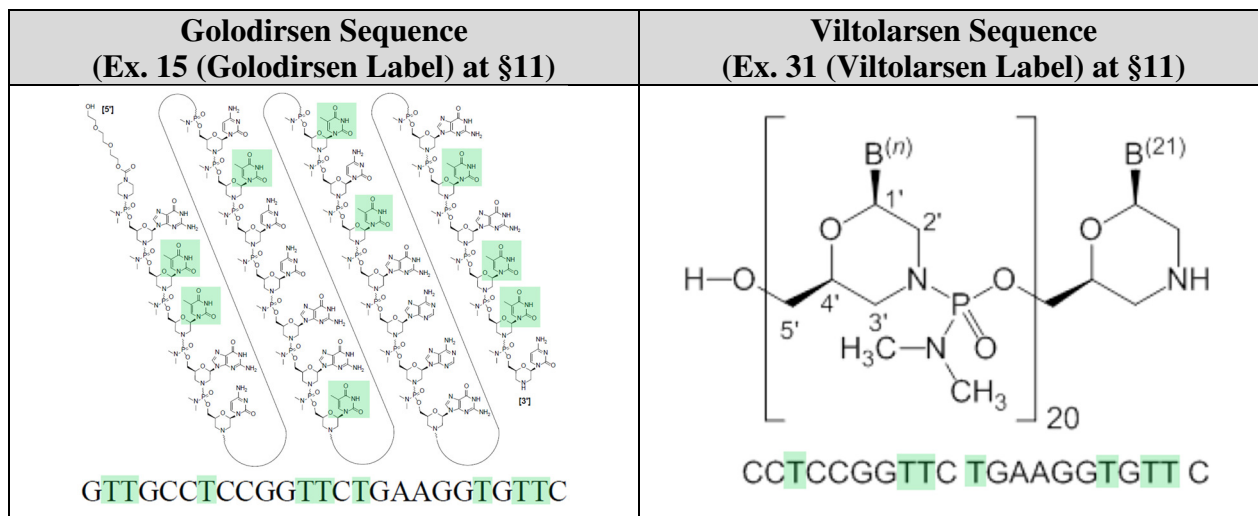
j)      (SEQ ID NO: 10)
      CXG XXG CCX CCG GXX CXG AAG GXG XXC XXG;

      (SEQ ID NO: 11)
k)      CAA CXG XXG CCX CCG GXX CXG AAG GXG XXC;
or

      (SEQ ID NO: 12)
l)      XXG CCX CCG GXX CXG AAG GXG XXC XXG XAC,
```

66. Dr. Hastings, however, omits the discussions that immediately follows: “One factor that can determine whether X is T or U is the chemistry used to produce the molecule. For example, if the molecule is a phosphorodiamidate morpholino oligonucleotide (PMO), X *will be* T as this base is used when producing PMOs. . . . Preferably, the base ‘X’ is only thymine (T).” Ex. 38 (the ’601 patent) at 6:19–26 (emphasis added). In other words, this patent instructs using only thymine bases when making a morpholino antisense oligonucleotide—which are also claimed in the Wilton patents. Consistent with this instruction, each morpholino oligomer empirically tested in the ’601 patent only contains thymine bases. *See id.* at cols. 10–11.

67. Consistent with this common practice in the art, both NS’s product and Sarepta’s Vyondys 53 (golodirsen) product only contain thymine bases (highlighted in green), illustrating the general practice in the art of making morpholino oligomers with thymine bases only.



68. Exhibit 40: Exhibit 40 is a review article published in 2022. *See* Ex. 40 (Helm 2022) at 1. Similar to Exhibit 38 above, it is unclear how this exhibit relates to what a skilled artisan in 2005 would have known. Further, Dr. Hastings states that this article describes “the advantages of using uracil and thymine in steric blocking antisense oligonucleotides.” Hastings Decl. ¶115 (citing Ex. 40 (Helm 2022) at 7). The quoted sentence reads: “C5’-methylations of

cytidine and uracil are used *throughout* the molecule in some steric block” antisense oligonucleotides. Ex. 40 (Helm 2022) at 7 (emphasis added). This section does not discuss using a mixture of uracil and thymine bases in a single antisense oligonucleotide. If anything, the quoted sentence suggests using thymine in place of uracil “throughout the molecule,” given that C5’-methylation of uracil converts it into thymine. *See id.*

69. Exhibit 41: Exhibit 41 is prescribing information for a drug known as Leqvio<sup>®</sup>. *See* Hastings Decl. ¶115. This drug was approved in 2021, many years after the Wilton patents were filed. Ex. 41 (Leqvio Label) at 1. Leqvio<sup>®</sup> is “a double-stranded small interference ribonucleic acid (siRNA), conjugated on the sense strand.” *Id.* at §12.1. While Dr. Hastings contends that this medication “includes both uracil bases and a thymine base” (Hastings Decl. ¶115), it should be noted that the antisense strand, i.e., the siRNA that is the active portion of the drug, only contains uracil bases. *See* Ex. 41 (Leqvio Label) at §11.

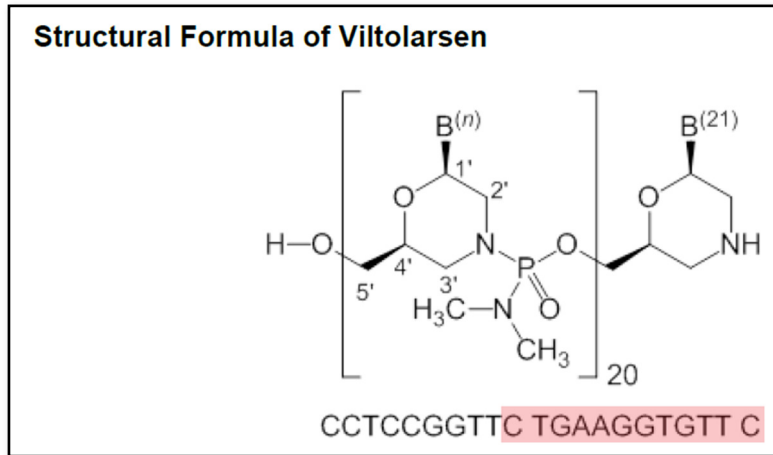
70. As Dr. Hastings acknowledges, “in many cases, oligonucleotides use only thymine or uracil bases—not both.” Hastings Decl. ¶106. This general practice, represented by an overwhelming majority of reported oligonucleotides having only thymine or uracil bases, further supports Sarepta’s construction, not Dr. Hastings’ construction and indefiniteness positions.

#### **4. Dr. Hastings’ “Theoretical” Examples Ignore NS’s Product**

71. In various places, Dr. Hastings presents “theoretical” antisense oligonucleotides containing uracil bases and thymine bases. According to Dr. Hastings, a skilled artisan would be unable to determine whether they fall inside or outside the scope of the claims. *Id.* ¶¶103–104, 109. I disagree. If the antisense oligonucleotide in question contains uracil bases, it is outside the scope of the claims.

72. While Dr. Hastings focuses on theoretical antisense oligonucleotides, she has not identified prior art oligonucleotides or exemplified oligonucleotides in the Wilton specification

containing both uracil bases and thymine bases. Notably, she also disregards the real-world example of NS's product. As shown below in **Figure 8**, NS's viltolarsen product only contains thymine bases, including in the portion derived from SEQ ID NO: 195 (identified by a red box). Thus, regardless of whether Sarepta's construction or Dr. Hastings' construction is adopted, NS's Viltepso® (viltolarsen) product meets the thymine bases limitation of the Wilton patents.



**Figure 8.** Annotated Structure of Viltolarsen  
(adapted from Ex. 31 (Viltolarsen Label) at §11)

**D. Term 2: “the target region is within annealing site H53A(+23+47) and annealing site H53A(+39+69)”**

Sarepta's Proposed Construction	NS's Proposed Construction
<p><i>Not indefinite</i></p> <p>Sarepta proposes construing the phrase as: “the target region is within nucleotides +23 to +69 of exon 53 of the human dystrophin pre-mRNA”</p>	<p><i>Indefinite</i></p>

### 1. The Annealing Site Phrase Identifies Nucleotides +23 to +69

73. In my Opening Declaration, I identified several reasons supporting Sarepta's construction of the annealing site phrase, including: (1) the known nomenclature system for defining the annealing site of an antisense oligonucleotide; (2) the prosecution history of the '851 and '827 patents; and (3) NS's statements in Europe. Op. Decl. ¶¶85–91. I explained that a skilled

artisan would understand this phrase to identify a region spanning from nucleotide +23 to nucleotide 69 of exon 53 of the human dystrophin pre-mRNA. *Id.* Read in context, the claims convey that the target region of the claimed antisense oligonucleotide falls *within* this discrete region. *Id.*

74. Dr. Hastings applies the same nomenclature system to the annealing site phrase, illustrating that a skilled artisan likewise would have been able to interpret the two recited annealing sites as nucleotides +23 to +47 and nucleotides +39 to +69, respectively. *See* Hastings Decl. ¶¶92–93. She acknowledges that the applicant during prosecution of the '827 patent explained that the annealing site phrase corresponds to a region “spanning from, and including, endpoint H53A+23 to, and including, endpoint H53A+69.”<sup>6</sup> Ex. 23 (Prosecution History Excerpt of the '827 patent) at SRPT-VYDS-0006276–77; Hastings Decl. ¶95 (the applicant “argued for the same interpretation as Sarepta and Dr. Stein”). But she does not address the statements from NS in Europe, interpreting “both” in the phrase “designated as annealing site H53A (+23+47), annealing site H53A (+39+69), or both” to mean “the area from nucleotide +23 until +69.” Ex. 11 (NS Notice of Opposition) at 4–5; Op. Decl. ¶¶90–91.

75. Dr. Hastings nevertheless disagrees with Sarepta’s construction. Hastings Decl. ¶93. Specifically, she rewrites the annealing site phrase as “‘within’ **both** annealing sites” and contends that the phrase must be limited to the 9 nucleotides shared by these two annealing sites

---

<sup>6</sup> Dr. Hastings does not address the statement in the prosecution history of the '851 patent explaining this phrase. Ex. 22 (Prosecution History Excerpt of the '851 patent) at SRPT-VYDS-0004786 (the applicant explaining that the claimed invention includes, for example, “the exon 53 target region +23 to +69”); Op. Decl. ¶89.

(nucleotides +39 to +47).<sup>7</sup> *Id.* She criticizes Sarepta’s construction as including nucleotides not shared by these two annealing sites, for example, nucleotides +23 to +38 and +48 to +69. *Id.*

76. By inserting “both” into the claim term, Dr. Hastings creates a new limitation that the target region must be physically present in *both* annealing sites. But the claims do not refer to “both” annealing sites. Instead, the claims only require that the target region is “within H53A(+23+47) and H53A(+39+69),” i.e., within nucleotides +23 to +69. Further, read in context, it is appropriate to read the term “within” to refer to a range of nucleotides in exon 53 of the human dystrophin pre-mRNA *within* which the target region must fall, rather than, as Dr. Hastings suggests, a requirement that the target region is within both annealing sites. Dr. Hastings bases her opinion on a word that is not present in the claims.

77. Moreover, Dr. Hastings does not account for other claim limitations that weigh against her interpretation. Importantly, the claims require that the base sequence must comprise “at least 12 consecutive bases” from SEQ ID NO: 195, which is 100% complementary to nucleotides +23 to +47 of exon 53. Ex. 1 (the ’851 patent) at claim 1; *see also id.* at Table 1A (SEQ ID NO: 195 corresponds to H53(+23+47) antisense oligonucleotide). Reading the annealing site phrase in context, a skilled artisan would have recognized that the phrase was not intended to limit the target region to only 9 nucleotides, because the target region *must* also contain at least 12 nucleotides that are 100% complementary to SEQ ID NO:195. To avoid this impossible conflict, a skilled artisan would have read the phrase in an inclusive manner, i.e., as covering nucleotides +23 to +69.

---

<sup>7</sup> Notably, Dr. Hastings’ articulation of “both annealing sites” phrase is the same phrase that NS interpreted to cover nucleotides +23 to +69 in Europe. Ex. 11 (NS Notice of Opposition) at 4–5.

78. Focusing on a few isolated statements from my Opening Declaration, Dr. Hastings contends that it is unclear whether Sarepta's construction places the claimed target region "within nucleotides +23 to +69" or requires it to cover the entire region spanning from +23 to +69. Hastings Decl. ¶¶93. Clearly, the disputed phrase uses the word "within" and Sarepta's construction likewise uses "within." Even the portion from my Opening Declaration that Dr. Hastings reproduced makes this clear. As I explained, "these overlapping annealing sites define a target region within exon 53," i.e., one present in "a region spanning from nucleotide +23 to nucleotide +69 of exon 53." *See id.* (reproducing Op. Decl. ¶¶88).

## **2. Dr. Hastings' Indefiniteness Positions Are Unreasonable**

79. Dr. Hastings' indefiniteness positions hinge on the same unsupported proposition that a skilled artisan: (1) would have understood that the defined target region spans nucleotides +39 to +47 and (2) "would struggle to understand the scope of the claim with reasonable certainty" because an antisense oligonucleotide cannot be limited to 9 nucleotides while concurrently having at least 12 nucleotides from SEQ ID NO: 195. Hastings Decl. ¶¶92. I disagree with her predicate, as a skilled artisan would not have identified the "target region" as spanning only nucleotides +39 to +47.

80. Other than inserting "both" in the phrase, Dr. Hastings does not explain how the claims or specification supports her interpretation. *See id.* ¶¶92–93. At most, she points to the fact that the Examiner once rejected the annealing site phrase by interpreting the phrase to cover 9 nucleotides only. *Id.* ¶¶94–95. But as she acknowledges, the applicant subsequently explained that the annealing site phrase corresponds to a region "spanning from, and including, endpoint H53A+23 to, and including, endpoint H53A+69." *Id.*; Ex. 23 (Prosecution History Excerpt of the '827 patent) at SRPT-VYDS-0006276–77. In other words, the applicant explained precisely what the phrase covers. Dr. Hastings provides no reason to dismiss the applicant's statement.

81. A skilled artisan would not have adopted Dr. Hastings' interpretation. As discussed above, reading the annealing site phrase to cover only 9 nucleotides creates an obvious conflict with other claim limitations. *See supra* ¶¶77. A skilled artisan would have recognized this conflict and understood that her interpretation conflicts with the language of the claims as a whole.

82. Dr. Hastings' interpretation is also inconsistent with the specification. None of the antisense oligonucleotides disclosed in the specification that are directed to exon 53 has a target region confined to the 9 nucleotides she identifies. *See* Ex. 1 (the '851 patent) at Table 1A.

83. Notably, Dr. Hastings does not state that the claims are indefinite under Sarepta's construction. *See* Hastings Decl. ¶¶91–95.

I declare that all statements made herein of my knowledge are true, and that all statements made herein on information and belief are believed to be true. These statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Date: February 27, 2023

By: Aaron Stein M.D. Ph.D.  
Cy Aaron Stein, M.D., Ph.D.

# Appendix C

**Materials Considered**

<b>Exhibit Number</b>	<b>Description</b>
1	U.S. Patent No. 9,994,851
2	U.S. Patent No. 10,227,590
3	U.S. Patent No. 10,266,827
4	Aartsma-Rus et al., “Targeted Exon Skipping as a Potential Gene Correction Therapy for Duchenne Muscular Dystrophy,” <i>Neuromuscul. Disord.</i> (2002) 12: S71-S77 (“Aartsma-Rus 2002”)
11	Nippon Shinyaku Co. Ltd., Opposition Against European Patent No. 2206781 B1 (“NS Notice of Opposition”)
15	Vyondys 53 <sup>®</sup> (Golodirsen) Prescribing Information (Revised: 2/2021) (“Golodirsen Label”)
17	Interference No. 106,007, Decision dated May 12, 2016
19	Mann et al., “Improved Antisense Oligonucleotide Induced Exon Skipping in the Mdx Mouse Model of Muscular Dystrophy,” <i>J. Gene Med.</i> (2002) 4(6): 644-654
20	Prosecution History Excerpt of U.S. Application No. 15/273,772
22	Prosecution History Excerpt of U.S. Patent No. 9,994,851
23	Prosecution History Excerpt of U.S. Patent No. 10,266,827
24	Summerton et al., “Morpholino Antisense Oligomers: Design, Preparation, and Properties,” <i>Antisense Nucleic Acid Drug Dev.</i> (1997) 7(3): 187-195 (“Summerton 1997(a)”)
31	Viltepso <sup>®</sup> (Viltolarsen) Prescribing Information (Revised: 3/2021) (“Viltolarsen Label”)
38	U.S. Patent No. 8,084,601 (“the ‘601 patent”)
39	Gene Tools, LLC, <i>Morpholino History, Production, and Properties</i> , available at <a href="https://www.gene-tools.com/history_production_and_properties">https://www.gene-tools.com/history_production_and_properties</a> (last accessed Feb. 6, 2023)
40	Helm, J., <i>Towards Personalized Allele-Specific Antisense Oligonucleotide Therapies for Toxic Gain-of Function Neurodegenerative Diseases</i> , Pharmaceutics 2022, 14, 1708
41	LEQVIO Prescribing Information, Revised 12/2021
48	Havens et al., “Targeting RNA Splicing for Disease Therapy,” <i>WIREs RNA</i> (2013) 4(3): 247-266 (“Havens 2013”)
49	Havens et al., “Splice-Switching Antisense Oligonucleotides as Therapeutic Drugs,” <i>Nucleic Acids Res.</i> (2016) 44(14): 6549-6563 (“Havens 2016”)
50	Takeda et al., “Exon-Skipping in Duchenne Muscular Dystrophy,” <i>J. Neuromuscul. Dis.</i> (2021) 8(s2): S343-S358 (“Takeda 2021”)
51	Merriam-Webster, Inc., <i>Merriam-Webster’s Collegiate Dictionary</i> 1341 (10th ed. 2000) (“Merriam-Webster Dictionary”)